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Stress in the interaction of the endophytic fungus *Piriformospora indica* with Arabidopsis

Dissertation

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Table of Contents

Summary

Piriformospora indica is an endophytic fungus and belongs to Sebacinales. It colonizes the roots of many plant species. This results in better plant performance and biotic and abiotic stress tolerance. Furthermore, the fungus enhances the biomass and seed yield. The symbiotic interaction of *P. indica* with the model plant *Arabidopsis thaliana* provides a good model system to investigate beneficial and non-beneficial traits in this relationship. I studied the effect of high fungal doses on the performance of *Arabidopsis* seedlings and found that growth of the seedlings is inhibited under these growth conditions, but photosynthetic parameters and the colonization rate is only little affected. Defense responses such as ROS production and the activation of defense genes are only moderately up-regulated. Overall, the results demonstrate that the roots can manage high fungal doses of *P. indica* in their environment without severe harm for the plant.

I investigated the response of the roots during early phases of the co-cultivation process, i. e. before a physical contact of the two partners has been established and the communication can only occur via chemical mediators. During a later phase when the fungal hyphae have reached the roots and started to colonize them, I observed a shift from mild defense against the microbe to the initiation of a mutualistic interaction. Based on microscopic, transcriptomic and physiological studies I showed that the defense response during the early phase of co-cultivation is mediated by chemical communication, since no physical contact has been established yet. When fungal hyphae reach the roots, the defense responses are down-regulated and physiological responses start to be activated which indicate a shift towards mutualism.

Finally, I established co-cultivation conditions with stress gradients. Seedlings were exposed to salt, osmotic and light stress gradients or to the pathogen *Alternaria brassicae*. I could demonstrate that a mild increase in the stress promotes the beneficial interaction between the two symbionts. The seedlings profit more from the interaction when they two symbionts are co-cultivated under mild stress.

Zusammenfassung

Piroformospora indica ist ein Pilz aus der Ordnung der Sebacinales, der zu endophytischem Wachstum fähig ist. Er kolonisiert die Wurzeln vieler Pflanzenarten. Dies resultiert in verbessertem Pflanzenwachstum und erhöhter Toleranz gegenüber biotischem und abiotischem Stress. Außerdem steigert der Pilz die Biomasse- und die Samenproduktion der Pflanzen. Die symbiotische Interaktion von *P. indica* mit der Modellpflanze *Arabidopsis thaliana* stellt ein gutes Modellsystem dar, um die nützlichen und weniger nützlichen Eigenschaften dieser Beziehung zu erforschen. Ich habe den Effekt hoher Pilzdosen auf das Wachstum von Arabidopsis-Keimlinge studiert und dabei herausgefunden, dass das Wachstum der Keimlinge dadurch inhibiert wird, jedoch die photosynthetischen Parameter sowie die Kolonisierungsrate unbeeinflusst bleiben. Verteidigungsreaktionen, wie die Produktion reaktiver Sauerstoffspezies und die Aktivierung von Verteidigungsgenen, sind nur leicht hochreguliert. Zusammengefasst demonstrieren diese Ergebnisse, dass die Wurzeln mit hohen Dosen von *P. indica* in ihrer Umwelt umgehen können, ohne dass die Pflanze ernsthaft beeinträchtigt wird.

Ich habe die Reaktion der Wurzeln während der frühen Phasen der Co-Kultivierung untersucht, zu einem Zeitpunkt da ein physikalischer Kontakt zwischen beiden Interaktionspartnern noch nicht erfolgt ist und die Kommunikation nur auf chemischer Ebene stattfinden kann. In der späteren Phase, wenn die Pilzhyphen die Wurzeln erreicht haben und beginnen diese zu kolonisieren, konnte ich den Wechsel von einer schwachen Verteidigungsreaktion gegen den Mikroorganismus zu dem Beginn einer mutualistischen Interaktion beobachten. Basierend auf mikroskopischen, transkriptomischen und physiologischen Untersuchungen konnte ich zeigen, dass die Verteidigungsreaktion während der frühen Phase der Co-Kultivierung durch chemische Kommunikation vermittelt wird, da noch kein physikalischer Kontakt besteht. Wenn die Pilzhyphen die Wurzeln erreichen, werden die Verteidigungsreaktionen herunterreguliert und physiologische Reaktionen werden aktiviert, was den Wechsel hin zum Mutualismus zeigt.

Schließlich habe ich Bedingungen für die Co-Kultivierung mit Stressgradienten etabliert. Keimlinge wurden dazu Salzstress, osmotischem Stress, Lichtstress oder dem Pathogen *Alternaria brassicae* ausgesetzt. Dabei konnte ich zeigen, dass eine leichte Erhöhung des Stresses die nützliche Interaktion zwischen den beiden Symbionten fördert. Die Keimlinge profitieren stärker von der Interaktion, wenn die zwei Symbionten unter leichtem Stress co-kultiviert werden.

1. Introduction

1.1. Plant microbe interaction

Interaction of plants with other organisms especially microorganism is unavoidable in their natural complex habitat. In biological ecosystems, symbiosis is an obligatory or facultative interaction between two species with beneficial or harmful effects for the involved partners. Symbiotic interaction occurs in different mutual and non-mutual ways; mutualism in which both individuals benefits from each other, and commensalism with benefits only for one organism without affecting the other partner and finally parasitism, when one partner (parasite) benefits while the other partner (host) gets harmed (Kogel et al. 2006,Oelmüller et al. 2009).

The valuable carbon source in plants is the main reason for microorganisms, in particular fungi to have mutualistic relationships. As a common benefit of this symbiotic interaction, plants also can acquire higher tolerance to biotic and abiotic stresses and subsequently increase their fitness and gain more growth promotion (Johnson and Oelmüller, 2009). The well-known examples of mutualism are Rhizobium bacteria that help plants to fix nitrogen and arbuscular mycorrhizal fungi (AMF) that increase uptake of water and nutrients (Redecker et al. 2000). According to estimations, only 20% of terrestrial plants do not form interaction with mycorrhiza (van der Heijden et al. 1998). Specifically in poor and dry soils, plant interactions with AM fungi are critical for their survival (Gemma et al. 2002). Stresses, nutrition conditions of interacting environment and genetic parameters of the plant and symbiont can results in shifting mutualism toward parasitism (Johnson and Oelmüller 2009, and references therein).

Biotic and abiotic stresses and their crop losses effects in agriculture are one of human civilization challenges that should be overcome in less than 40 years, before human population reaches to 10 billion. Biological based methods to protect plants against stresses are human's best options with very less environmental consequences. Among them symbionts that increase plant tolerance against abiotic stresses and protect them against biotic stresses play a critical role (Dawson and Hilton, 2011, Powles and Yu, 2010). Study of AMF interaction with plant partners is limited because of the dependency of AMF to the living hosts. A promising alternative for a microorganism, which colonizes plant roots and grows on artificial media, is *Piriformospora indica*. In combination with the model plant *Arabidopsis thaliana*, it provides a unique system to study different molecular and physiological aspects of fungal symbiotic interaction with plants.

1.2. Interaction of *Piriformospora indica* with plants

The basidiomyceteous fungus *Piriformospora indica* is a member of Sebacinales that produces typical pear-shaped chlamydospores and colonizes the root cortex (Fig. 1).

It was originally isolated from India and have been found in Pakistan, Philippines, Australia, Portugal, Italy and several South American countries (Varma et al. 2012). Its cell wall structure is very thin including several layers that form multinucleate and regularly septate hyphae (Kost and Rexer 2013). Chlamydospore cytoplasm contains 8-25 nuclei. Root colonization by *P. indica* has been documented for different plant species (Oelmüller et al. 2009, Qiang et al. 2012, Lahrmann and Zuccaro 2012). The fungus increases seed yield in colonized plants (Oelmüller et al. 2009, Shahollari et al. 2007, Sherameti et al. 2005, Vadassery et al. 2009, Waller et al. 2005, Zuccaro et al. 2011), enhances metabolism, nitrate and phosphate uptake (Sherameti et al. 2005, Shahollari et al. 2005, Yadav et al. 2010) and increases abiotic stress tolerance (Sherameti et al. 2008, Baltruschat et al. 2008, Sun et al. 2010) as well as biotic stress resistance (Oelmüller et al. 2009, Stein et al. 2008).

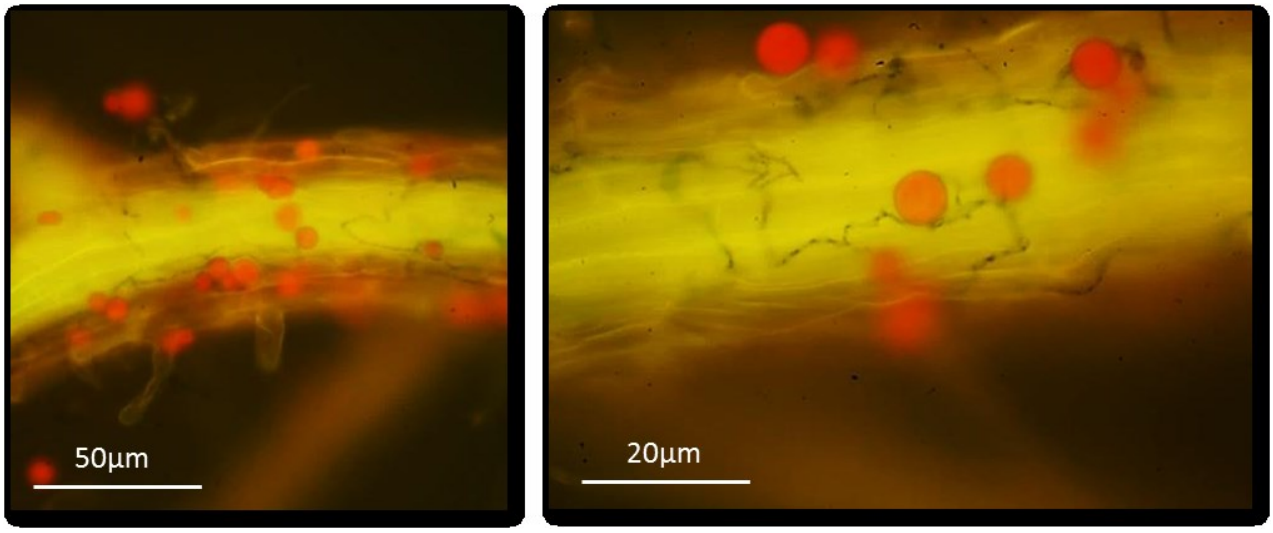

Fig 1.1. Arabidopsis root colonization by *Piriformospora indica.*

Chemical communication prior to the physical interaction has been reported as important step in microbe recognition in fungal and bacterial interactions with plant hosts (Vadassery et al. 2009, Oldroyd and Downie 2008, Kosuta et al. 2003). Chemicals released by microbes like mycorrhi-

zal fungi, Myc factors and factors from rhizobial bacteria (Nod factors) result in reprograming of gene expression in their hosts (Satoh et al. 2013). In addition, they overcome the immune system of the host as well (Kloppholz et al. 2011).

A beneficial symbiotic relationship is the consequence of a balanced interaction between two partners (Kogel et al. 2006, Paszkowski et al. 2006, Johnson and Oelmüller 2009). This can be determined via an signaling-based equilibrium of plant defense and fungal growth (Kogel et al. 2006, Paszkowski et al. 2006, Oldroyd 2013, Nehls et al. 2010, Campos-Soriano and Segundo 2011, León-Morcillo et al. 2012, Fester and Hause 2005 , Harrison 2005, Jung et al. 2012, Campos-Soriano et al. 2010). Exchange of soil nutrients and the plant carbon status are the basis of mutualistic interactions in mycorrhiza (Harrison 2012). Nevertheless, the level of root colonization can shift in this interaction from mutualism to parasitism or the other way round (Tanaka et al. 2006) which can be regulated by fungi (Campos-Soriano and Segundo 2011) or by plants (Nehls et al. 2010, León-Morcillo et al. 2012).

When symbiosis is established in the *P. indica* and plant interaction, defense and stress genes are mainly deactivated and the level of reactive oxygen species (ROS) is low in hosts (Johnson and Oelmüller 2009, Sherameti et al. 2008, Camehl et al. 2011). Also, it has been shown that *P. indica* suppresses host defense (Jacobs et al. 2011). In comparison with mycorrhizal fungi, study of the plant - *P. indica* interaction system is much easier because *P. indica* can grow on synthetic media (Varma et al. 1999).

1.3. Role of *P. indica* in plant tolerance against abiotic and biotic stress

Natural habitats of plants bring different biotic and abiotic stress challenges. Main biotic stress challenges are from microbes (pathogenic fungi, bacteria and viruses), insects, and other herbivores. Abiotic stresses like salinity, drought, light, heavy metals, nutrient deficiency and temperature stresses cause serious limitation in plant growth. Plants use different strategies to bear biotic and abiotic stresses. Symbiosis with microbes is an efficient way for enhancing the resistance to the biotic stresses and higher tolerance against abiotic stresses. *P. indica* interaction with Arabidopsis and Chinese cabbage roots results in higher growth under drought stress (Sherameti et al. 2008, Sun et al. 2010). Furthermore, it enhances stress tolerance by increasing the level of anti-

oxidants in the colonized plants (Foyer and Shigeoka 2011, Baltruschat et al. 2008). Also up-regulation of *MDAR2* and *DHAR5,* ascorbate reductase genes, were documented in both shoots and roots of Arabidopsis plants interacting with *P. indica* in drought stress conditions (Vadassery et al. 2009). Plant protection against biotic stresses (e.g. by *Blumeria graminis* or *Fusarium culmoru*) has been reported in barley colonized with *P. indica* (Waller et al. 2005). *P. indica* induced resistance against biotic stresses has been shown for *Triticum aestivum* against *Pseudocercosporella herpotrichoides*, for *Zea mays* against *Fusarium verticillioides*, *Solanum lycopersicum* and for tomato against *Verticillium dahlia*e, *etc*. (Serfling et al. 2007, Kumar et al. 2009, Fakhro et al. 2010).

Induced systemic resistance in barley via strong up-regulation of defense-related genes against powdery mildew, a leaf pathogen, is just one of the outcomes of *P. indica* interaction with barley roots (Molitor et al. 2011). ROS levels increase and antioxidant synthesis is a natural plant response to abiotic stresses (Waller et al. 2005, Serfling et al. 2007, Kumar et al. 2009).

Studies of knock out plants for *NahG*, *NPR1*, *JAR1* genes revealed that JA signaling is important in plant-induced resistance due to root colonization by *P. indica* (Stein et al. 2008). In contrast with fungal pathogens, a few reports showed that plants colonized by endophytes are more susceptible to pests (Barazani et al. 2005). For instance, the spreading virus rate is higher in colonized tomatoes by endophytes in low light condition (Fakhro et al., 2010).

P. indica is described to have helpful effects on plant growth in different plant species (Shahollari et al. 2007, Vadassery et al. 2009, Camehl et al. 2011, Nongbri et al. 2012 a and b, Jogawat et al. 2013, Ye et al. 2014, Lahrmann et al. 2013). The fungus improves uptake of required nutrition in plants through affecting the transcriptome in the host (Sherameti et al. 2005, Yadav et al. 2010) which consequently results in resistance against biotic and abiotic stress (Varma et al. 1999; Waller et al. 2005; Baltruschat et al. 2008; Stein et al. 2008). It promotes the growth of shoot and root by accumulation of secondary metabolites and local systemic resistance induction (Sirrenberg et al. 2007). It is believed that the degree of root colonization is controlled by the plant, and it determines the interaction balance between both partners (Sherameti et al. 2008; Khatabi and Schäfer 2012).

1.4. Biotic stresses

Biotic stresses result in crop losses in different commercial plants each year. Management of biotic stress costs several billion dollars per year and conventional methods have extra expenses by effecting the environment and human health (Klosterman et al. 2009). Biological based methods of controlling biotic stresses are a convenient option for the human society. Among them, symbiotic interacting microbes provide a unique opportunity to induce plant resistance/tolerance against biotic stress (Berg et al. 2001, Tjamos et al. 2005, Li et al. 2012). In this study, we investigate the effect of *P. indica* on the plant´s response to pathogen.

1.4.1. *Verticillium dahliae,* a root pathogen

Verticillium is a typical hemibiotroph fungus with an initial phase in root xylem (biotrophic phase). The hyphae penetrate inter- and intracellularly into the central root cylinder (Reusche et al. 2013) (Fig. 2). This phase is followed by a necrotrophic phase in shoots (Klosterman et al. 2011).

Production of conidia and microsclerotia in the xylem vessels disturbs water transportation in the plants that is visible as wilt symptom (Pegg and Brady, 2002). In addition, *Verticillium* phytotoxins and lytic enzymes induce disease symptoms in cotton, tomato, tobacco, olive tree or Arabidopsis (Buchner et al. 1989).This vascular wilt pathogen infects different plant species from temperate to subtropical climates including important agricultural and horticultural crops and ornamental plants (Pegg and Brady 2002; Fradin and Thomma 2006; Klosterman et al. 2009). Dehydration, chlorosis, necrosis and vein clearing are some typical disease symptoms. Isolation of Verticillium-resistant cultivars is crucial because of the resistance to conventional fungicides, presumably the consequence of microsclerotia formation in the Verticillium life cycle (Klosterman et al. 2009). Several resistance genes play important roles in resistance against *Verticillium* including the *Ve* gene which provides resistance against race 1 isolates of *V. dahliae* in tomato (Kawchuk et al. 2001, Fradin et al. 2009), genes involved in cytokinin biosynthesis (Reusche et al. 2013), *EWR*1 (Yadeta et al. 2014), *RabGAP*22 (Roos et al. 2014), *GbSTK* (Zhang et al. 2013), genes for the glucosinolate biosynthesis (Witzel et al. 2013), *Gbve*1 (Zhang et al. 2012), *AHL*19 (Yadeta et al. 2011), *PevD*1 (Bu et al. 2014) and *NaD*1 (Gaspar et al. 2014). Furthermore, it has

been shown that rhizosphere bacteria play an antagonistic role against *V. dahliae* (Berg et al. 2001, Li et al. 2012) by inducing antibiosis, parasitism, and induced resistance in host plants (Tjamos et al. 2005).

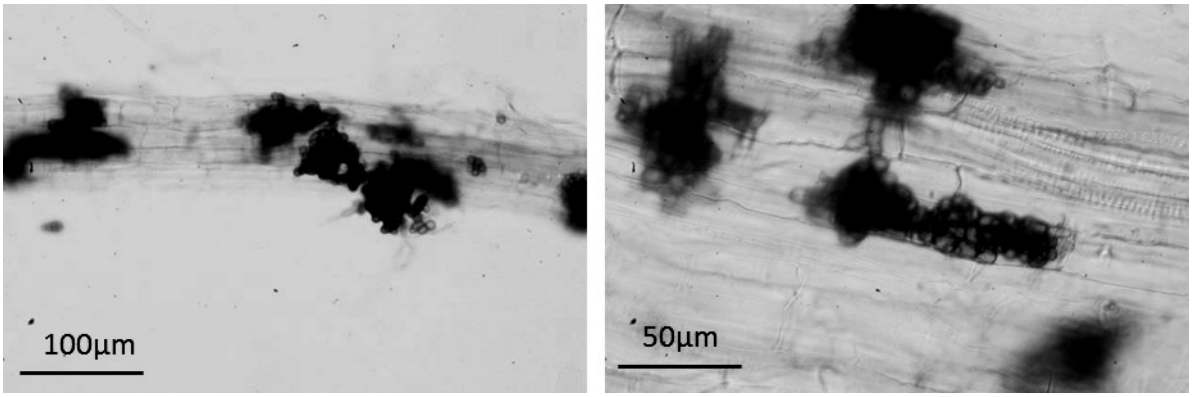

Fig 1.2. Infection of Arabidopsis roots by *Verticillium dahliae.*

1.4.2. Leaf pathogen *Alternaria brassicae*

A. brassicae, the causal agent of black spot (Fig 3) is one of the most destructive pathogen in *Brassica* spp. worldwide (Bains and Tewari 1987, Tewari and Bains 1997). *A. brassicae* has been reported as serious pathogen for *B. rapa*, *B. napus* and *B. juncea* cultivations (Saharan 1993) and cultivated crucifers (Guillemette et al. 2004). The chlorotic margins and gray, brown or black lesions in leaves, stems, and siliques are the main symptoms of black spot (Tewari and Bains1997).

Alternaria blight is another important disease caused by *Alternaria* in mustard (*Brassica juncea*) (Mondal et al. 2003). Alternaria species produce host-specific or host-selective toxins (HSTs), which play important roles in fungal pathogenicity. HSTs act at early phase of pathogenicity because it is produces during germination of fungal spores on the host surface (Parada et al. 2008) *A. brassicae* produces four low molecular weight cyclodepsipeptide phytotoxins named destruxins (Buchwaldt and Green 1992, Tewari and Bains1997) and the major phytotoxins is Destruxin B (Tewari and Bains1997).

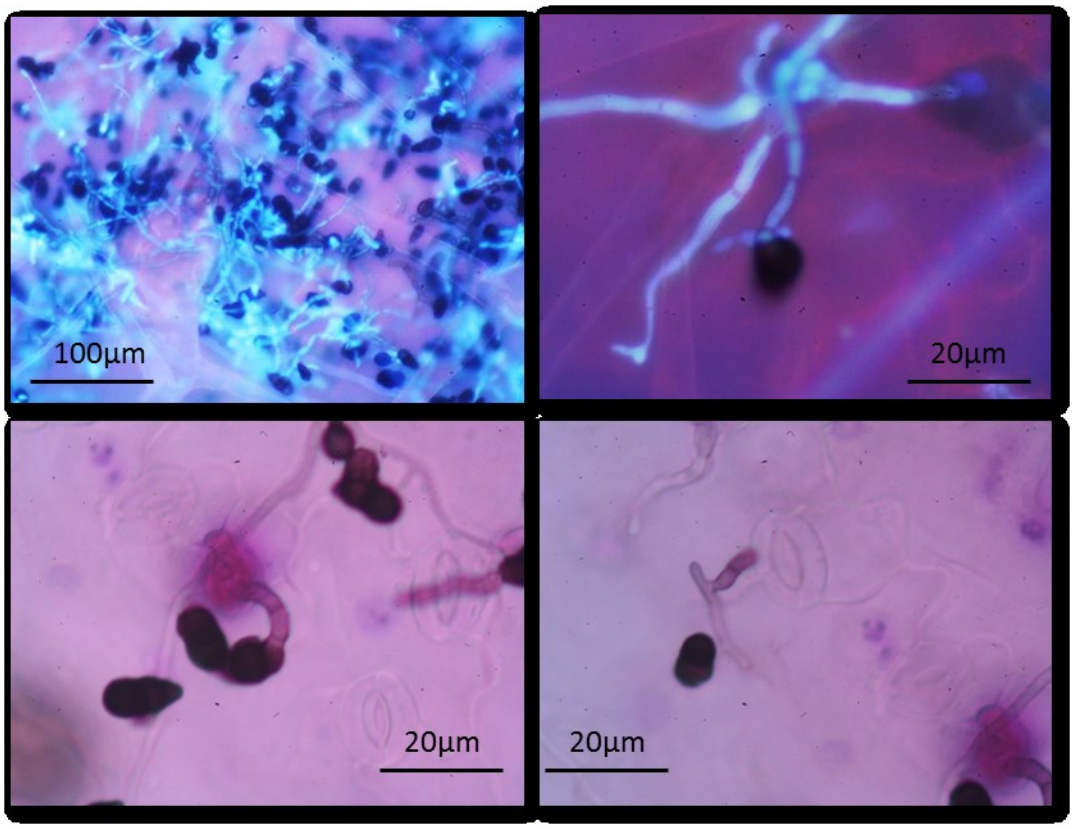

Fig 1.3. Infection of Arabidopsis leaves by *Alternaria brassicae*.

Control of disease by chemical fungicides is normally not sufficient and transgenic lines have been suggested to overcome the *A. brassicae* damage in agriculture (Mondal et al. 2007). In addition, finding biological methods to increase plant resistance against Verticillium and Alternaria is another opportunity in protecting plants from pathogens.

1.5. Abiotic stress effects on plants

Nutrition deficiency, ion toxicity, hormonal imbalance, defense response and defects of plant performance are main stresses effects on the plant (Nadeem et al. 2013). Plant osmotic potential is very sensitive to water deficiency and its modification reduces plant growth and consequently decreases plant yield (Kramer and Boyer 1995, Feng et al. 2002, Ruiz-Lozano 2003). Salinity of the soils increases oxidative stress (Gill and Tuteja 2010) by enhancing the level of hyperionic and hyperosmotic stresses (Vaidyanathan et al. 2003, Ramoliya et al. 2006, Porcel et al. 2012) and results in deficiency of phosphorus (P), NO_3^-, Ca^{2+} and K^+ (Vicente-Sánchez et al. 2013).

Reduced chlorophyll content is also a consequence of Mg^{2+} deficiency (Giri et al. 2003). About 2% of the plant dry weight is P and it is essential for development and regulation of key metabolic pathways and an important part of nucleic acids, ATP and membrane phospholipids of plants. Moreover, P is involved in energy generation, nucleic acid synthesis, photosynthesis, glycolysis, respiration, membrane synthesis and stability, enzyme activation/inactivation, redox reactions, signaling, carbohydrate metabolism and nitrogen fixation (Abel et al. 2002, Vance et al. 2003). Pi is below the optimal plant requirement (usually 10 mM) and poorly mobile for plants from soil (Batjes 1997). Competition for Pi uptake by some microbial species and its conversion into organic forms reduce solvable Pi that plant are able to uptake (Hinsinger 2001, Jia et al. 2011, Karthikeyan et al. 2007, Lambers et al. 2011, Lei et al. 2011, Lloyd and Zakhleniuk 2004). The deficiency of P in 5.7 billion hectares of agricultural land can mitigate by application of fertilizers or by soil microorganisms like plant growth-promoting rhizobacteria or AMF. In addition, plants gradually deplete the area around roots from nutrients by absorption, and they spend energy to expand the root system into the rich areas (Nadeem et. al. 2013). The excessive application of fertilizers has environmental side effects, results in water body enrichment with nutrients and subsequently causes eutrophication and toxic algal blooms (López-Arredondo and Herrera-Estrella 2012, Downing et al. 2001). The plant reaction to the nutrient deficiency is energy consuming and observed as increase of the root hair number and size, and secretion of organic acids to increase solubility of nutrients (Marschener 1998; Khan et al. 2000).

Plants take advantage of symbiotic interaction to overcome the stress (Nadeem et. al. 2013). For example, interaction with AMF causes an increase in uptake of P through fungi (Bever et al. 2001, Balzergue et al. 2011). Fungi can reach nutrient-enriched area, transfer nutrients toward roots with lower energy consumption than the plants and effectively improve plant performance (Nadeem et. al. 2013)**.** Symbiotic fungi also facilitate access to water for the plant via hydraulic conductivity improvement (Rosendahl and Rosendahl 1991, Giri et al. 2003). Under water stress, plants use symbiosis as one of effective mechanisms for tolerance (Sylvia et al. 1993, Subramanian et al. 1995, Brown and Bethlenfalvay 1988, Subramanian et al. 2006, Augé 2001), balance the osmotic potential (Azco´n et al. 1996, Goicoechea et al. 1998, Ruiz-Lozano 2003, Ruiz-Lozano et al. 2006) and increase the water plant potential (Subramanian et al. 2006). Also in heavy metal stress, fungal interaction with roots results in detoxification of the root and mycelia environment by extracellular reduction of the metal ions and subsequent conversion into insolu-

ble forms (Berreck and Haselwandter 2001, Hildebrandt et al. 2007, Vahabi et. al. 2011, Vahabi and Karimi 2014, Feng et al. 2013). Plants can also overcome salt stress via antioxidant production (Sekmen et al. 2007, Garg and Manchanda 2009, Turkan and Demiral 2009, Manchanda and Garg 2011, Ruiz-Lozano et al. 2012)

PiPT, a fungal high-affinity Pi transporter in *P. indica,* increases the transfer of Pi to the roots (Yadav et al. 2010, Kumar et al. 2011). Its expression depends on the amount of solvable Pi in the soil and is stimulated in P-deprived environments that results in higher growth promotion rate in P-depleted areas compared to P-rich conditions (Kumar et al. 2011). In return, *P. indica* colonization enhances the expression of the nitrate reductase and starch-degrading enzyme *SEX1* genes in roots of *A. thaliana* (Sherameti et al. 2005).

1.6. Plant hormones

Five classes of hormones in plants, auxin, cytokinin, ethylene (ET), abscisic acid (ABA) and gibberellins, play important roles in plant growth, development, cell division, elongation and differentiation (Weier and Rost, 1979). Also jasmonic acid (JA) and salicylic acid (SA) are involved in the plant defense and interaction with different microorganisms (Foo et al. 2013a). Hormones and their derivatives have multiple functions. Depending on the developmental stage of the plant, action site and concentration, hormones also participate in stress tolerance. Auxin, ET, cytokinin and strigolactone are involved in root architecture adjustment under P deficiency (Stepanova and Alonso 2009, Lynch and Brown 1997, Kapulnik et al. 2011). Phytohormone regulation determines the root architecture, gravitropism, parenchyma formation and expression level of root phenotype related genes.

Phototropism and gravitropism, plant architecture, organ patterning, vascular development and growth are some of the processes mediated by auxin (Davies 1995). It is also involved in root branching after colonization in some plant species by AMF (Gianiniazzi-Pearson et al. 1996, Foo 2013b), but not in all species (Jentschel et al. 2007, Campanella et al. 2008, Zsögön et al. 2008). It has been documented that auxin is required for AM infection, but not for development and formation of fungal structures and arbuscules after colonization. Auxin is involved in root clusters and lateral root architecture during P deficiency (Bates and Lynch 1996, Nacry et al. 2005, Jain et al. 2007). Development of lateral roots depends on auxin transport and sensitivity to low

P conditions (Nacry et al. 2005). Auxin correlation with low concentration of P has been demonstrated in white lupine roots (Gilbert et al. 2000; Vance et al. 2003; Marchive et al. 2009).

Under optimal P concentration, ET inhibits root elongation but induces it under low P condition (Borch et al. 1999). The effect of ET in plant-fungi symbiotic interaction under biotic and abiotic stress conditions remains unclear. Involvement of ET, SA and JA in response to stresses is well known. There is no clear explanation for the role of ET in AMF-plant interaction yet. ET-overproduction in the *epinastic* and *never ripe* mutants as well as in ET-insensitive mutants affects the AM colonization in tomato (Azcon-Aguiler et al. 1981, Ishii et al. 1996, Geil et al. 2001, Zsögön et al. 2008, de Los Santos et al. 2011), but the results have been considered as contradictory (de Los Santos et al. 2011). It has also been described that *etr1*, *ein2*, *ein3* and *eil1*, ET biosynthesis related genes, promote the symbiotic interaction of *P. indica* and Arabidopsis. Mutation in *etr1, ein2,* and *ein3/eil1* double mutation results in reduction of the symbiotic effect of the fungus on plant growth (Camehl et al. 2010). However, much more is known about effects of JA in the promotion of AM colonization (Regvar et al. 1996, Landgraf et al. 2012). JA deficiency in the tomato *spr2* mutant shows lower colonization rate by AMF (Li et al. 2003). Contradictory results have been reported for higher JA levels which resulted in lower colonization of *Tropaeolum majus* and *Carica papaya* (Ludwig-Müller et al. 2002). Higher colonization of tomato roots have been seen in the JA-insensitive *jar-1* mutant, which also showed, reduced root colonization by exogenous JA (Herrera-Medina et al. 2008). It seems that the role of JA in AMF colonization is a species-, hormone dose-, timing- and nutrition-dependent process.

SA responses to pathogen attack plays an important role in plant defense reactions (Foo et al. 2013a) but it is not clear if it is involved in AM formation in plant roots. However, the hormone is important in early colonization stages by AM (Herrera-Medina et al. 2003, Blilou et al. 1999).

Cytokinins are involved in the induction of the growth of the aerial part and reduces root growth (Aloni et al. 2006) while during P deficiency cytokinin reduces root growth and subsequently results in the formation of lateral roots (Horgan and Wareing 1980, Kuiper et al. 1988). CYTOKININ RESPONSE1 (CRE1), a cytokinin receptor, is down-regulated during P deficiency stress (Franco-Zorrilla et al. 2002) and exogenous cytokinin down-regulates Pi uptake transporter-related genes (Brenner et al. 2005; Martin et al. 2000). Like other hormones, its role in the interaction of symbiotic fungi and plants under biotic and abiotic stresses is unknown.

1.7. Phytohormones in the mutualistic interaction with *P. indica*

Changes in the auxin, cytokinin, ET, abscisic acid and gibberellin levels have been reported during beneficial interaction of *P. indica* with different plant species including Arabidopsis, barley and Chinese cabbage (Camehl et al. 2010, Vadassery et al. 2009, Schäfer et al. 2009, Lee et al. 2011). Exogenous auxin induces colonization of host plants by AM (Ludwig-Müller and Guther 2007). Vadassery et al. (2008) suggested that *P. indica*-induced growth promotion in *Arabidopsis* is independent of the auxin level because auxin mutants (*ilr1-1*, *nit1-3*, *tfl2*, *cyp79 b2b3*) responded to the fungus. Also, in *P. indica* interaction with Arabidopsis and Chinese cabbage the enhancement of the auxin level is important (Sirrenberg et al. 2007; Sun et al. 2010). The *AUX1* gene, encoding an auxin growth regulator, is presumed to be a target of *P. indica* in Chinese cabbage root colonization (Sun et al. 2010). Negative effects on growth promotion in *etr1*, *ein2* and *ein3/eil1* mutants during interaction suggested that ET has an important role in balancing the symbiotic interaction (Camehl et al. 2010). In addition, the involvement of gibberellins in *P. indica* – barley interaction has been shown by Schäfer et al. (2009). It seems that most plant hormones are involved in the beneficial plant/*P. indica* interaction but their effect on the interaction under biotic and abiotic stresses needs to be further studied.

2. Aim of the Project

The main goal of my study was to investigate the role of defense in the symbiotic interaction between *P. indica* and *Arabdiopsis thaliana* seedlings. Therefore, three co-cultivation conditions were analyzed:

1. I studied the effect of high fungal doses of *P. indica* on the performance of Arabidopsis seedlings. I was interested in understanding whether high fungal doses in the root environment affect the fitness of the plant and the balance between growth and defense.
2. Moreover, I investigated the effect of the fungus on the plant transcriptome and on physiological parameters during early phases of co-cultivation, which favor the establishment of a beneficial interaction. I have chosen an early time point (2 days after co-cultivation of the two symbionts) when a physical contact has not yet been established and the communication can only occur via chemicals released by the fungus into the medium, and compared this situation with a later time point (6 days after co-cultivation) when the hyphae have reached the roots. I investigated stress responses induced by *P. indica* in the roots at these two time points.
3. Furthermore, I developed co-cultivation conditions of the two symbionts in which *P. indica*-colonized plants were exposed to stress gradients. Using physiological, biochemical and molecular techniques, I investigated the role of the fungus on plant performance under different stress conditions. I was particularly interested in understanding whether an increase in stress affects the fungus-induced benefits for the plant and whether this has an influence of the balance between growth and defense.

3. Manuscript Overview

Manuscript I

Vahabi K, Camehl I, Sherameti I, Oelmüller R (2013) Growth of Arabidopsis seedlings on high fungal doses of *Piriformospora indica* has little effect on plant performance, stress, and defense gene expression in spite of elevated jasmonic acid and jasmonic acid-isoleucine levels in the roots. *Plant Signaling & Behavior* 8:e26301; PMID: 24047645; http://dx.doi.org/10.4161/psb.26301.

In this work, we conclude that growth of *A. thaliana* seedlings on high fungal doses of *P. indica* has little effect on the overall performance of plants, although elevated JA and JA-Ile levels in the roots induce a mild stress or defense response.
KV designed and carried out most of the experiments. IC and IS helped in data analysis. . KV, IS and RO wrote the article. RO supervised the research.

Manuscript II

Vahabi K, Sherameti I, Bakshi M, Mrozinska A, Ludwig A, Reichelt M, Oelmüller R (2015) The interaction of Arabidopsis with *Piriformospora indica* shifts from initial transient stress induced by fungus-released chemical mediators to a mutualistic interaction after physical contact of the two symbionts. *BMC Plant Biology* 15:58 doi:10.1186/s12870-015-0419-3.

Information exchange of the *P. indica* and Arabidopsis during short and long term interaction along with their effect on the plant root and shoot performance and physiological characters have been studied. We propose that exudated compounds of *P. indica* results in induction of stress and defense in Arabidopsis during short term interaction. As soon as a physical contact has been established defense related genes are down-regulated and genes involved in plant growth promoting, metabolism and performance are upregulated.
KV designed and carried out most of the experiments. IS, MB, AM and AL helped in transcriptome analysis. MR did the phytohormone analysis. KV, IS and RO wrote the article. RO supervised the research.

Manuscript III

Vahabi K, Karimi Dorcheh S, Monajembashi S, Sherameti I, Westermann M, Reichelt M, Falkenberg D, Hemmerich P, Oelmüller R (2015) Role of stress in Arabidopsis - *P. indica* interaction. Submitted to Mycorrhiza (manuscript submission: MCOR-D-15-00044)

In this study *P. indica* colonized Arabidopsis seedlings were grown during various levels of stresses. Our results showed that an increasing in the stress level enhances the symbiotic effect. KV designed and carried out most of the experiments. SK helped in data analysis. SC helped in the biophoton experiment, MR did the phytohormone analysis. DF helped in light stress experiment, MW did the SEM. KV, SK, IS and RO wrote the article. RO supervised the research.

I am co-author in the following manuscripts

Manuscript IV

Sun C, Shao Y, Vahabi K, Lu J, Bhattacharya S, Dong S, Yeh K-W, Sherameti I, Lou B, Baldwin I T, Oelmüller1 R (2014) The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection by down-regulation plant defense responses. *BMC Plant Biology* 14:268.

We conclude that *P. indica* is an efficient biocontrol agent that protects Arabidopsis from *V. dahliae* infection. Our data demonstrate that *V. dahliae* growth is restricted in the presence of *P. indica* and signals from *P. indica* must participate in the regulation of the immune response against *V. dahliae*.
CS designed and carried out most of the experiments. YS prepared the exudates from *V. dahliae*. KV did the root microscopy and long term experiment for *ein3* mutant in soil. JL contributed the phytohormones (JA, JA-Ile, SA, OPDA and ABA) analysis. SB did the ethylene measurement. IS helped to write the paper. BL and I. TB contributed to the discussion. The project was funded by a project of SD, K WY. and RO.

Manuscript V

Nongbri P L, Vahabi K, Mrozinska A, Seebald E, Sun C, Sherameti I, Johnson JM, Oelmüller R (2013) Balancing defense and growth Analyses of the beneficial symbiosis between *Piriformospora indica* and *Arabidopsis thaliana*. *Symbiosis DOI 10.1007/s13199-012-0209-8.*

In this review paper, we described the potential role of *P. indica* under abiotic and biotic. This article highlights the important biological and molecular features of the fungus and the potential biotechnological applications as a plant growth-promoting mycorrhizal fungus.

PLN, KV, AM, ES, CS, IS JMJ and RO have written this manuscript. ES and JMJ read the manuscripts and have given conceptual advice for improvement.

Some of techniques we developed have been published in following three technical notes

Manuscript VI

Vahabi K, Johnson JM, Drzewiecki C, Oelmüller R (2011) Fungal staining tools to study the interaction between the beneficial endophyte *Piriformospora indica* with *Arabidopsis thaliana* roots. *Journal of Endocytobiosis and Cell Research* 21:77-88.

In this technical note we describe different staining methods to study the histology of *P. indica-Arabidopsis thaliana* interaction.

KV designed and carried out most of the experiments. JMJ and CD did two staining methods. KV, JMJ, CD and RO wrote the article. RO supervised the research. All authors read and approved the final manuscript

Manuscript VII

Vahabi K, Meischner D, Oelmüller R (2014) Interaction of Arabidopsis and *Piriformospora indica* in a hydroponic system. *Journal of Endocytobiosis and Cell Research* 25: 63-65.

In this technichal note we designed a aquatic phase and controlable system for cocultivation of Arabidopsis seedlings with the beneficial root colonizing fungus *Piriformospora indica*.

KV designed and carried out most of the experiments. KV, DM and RO wrote the article. KV supervised the research.

Manuscript VIII

Vahabi K, Sun C, Govindaswamy J, Falkenberg D, Venus T, Oelmüller R (2015) Stomata staining in Arabidopsis. *Journal of Endocytobiosis and Cell Research* 26: 21-24.

In this technichal note we provide different and rapid stomata staining methods which could help to investigate the phenotypes under natural condition and even after pathogen attack which also can be used in quntification of open and closed stomata.

KV designed and carried out most of the experiments. JG, DF and TV participated in optimizing of the staning methodes. KV, SC and RO wrote the article. KV supervised the research. All authors read and approved the final manuscript

4. Manuscripts

4.1 Manuscript I

Vahabi K, Camehl I, Sherameti I, Oelmüller R (2013) Growth of Arabidopsis seedlings on high fungal doses of *Piriformospora indica* has little effect on plant performance, stress, and defense gene expression in spite of elevated jasmonic acid and jasmonic acid-isoleucine levels in the roots. *Plant Signaling & Behavior* 8:e26301; PMID: 24047645; http://dx.doi.org/10.4161/psb.26301.

RESEARCH PAPER

Plant Signaling & Behavior 8:11, e26301; November 2013; © 2013 Landes Bioscience

Growth of *Arabidopsis* seedlings on high fungal doses of *Piriformospora indica* has little effect on plant performance, stress, and defense gene expression in spite of elevated jasmonic acid and jasmonic acid-isoleucine levels in the roots

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Keywords: *Piriformospora indica*, mutualistic plant/microbe interaction, stress response, defense, jasmonic acid, jasmonic acid-isoleucine, H_2O_2

The endophytic fungus *Piriformospora indica* colonizes the roots of many plant species including *Arabidopsis* and promotes their performance, biomass, and seed production as well as resistance against biotic and abiotic stress. Imbalances in the symbiotic interaction such as uncontrolled fungal growth result in the loss of benefits for the plants and activation of defense responses against the microbe. We exposed *Arabidopsis* seedlings to a dense hyphal lawn of *P. indica*. The seedlings continue to grow, accumulate normal amounts of chlorophyll, and the photosynthetic parameters demonstrate that they perform well. In spite of high fungal doses around the roots, the fungal material inside the roots was not significantly higher when compared with roots that live in a beneficial symbiosis with *P. indica*. Fifteen defense- and stress-related genes including *PR2*, *PR3*, *PAL2*, and *ERF1* are only moderately upregulated in the roots on the fungal lawn, and the seedlings did not accumulate H_2O_2/radical oxygen species. However, accumulation of anthocyanin in *P. indica*-exposed seedlings indicates stress symptoms. Furthermore, the jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile) levels were increased in the roots, and consequently *PDF1.2* and a newly characterized gene for a 2-oxoglurate and Fe^{2+}-dependent oxygenase were upregulated more than 7-fold on the dense fungal lawn, in a JAR1- and EIN3-dependent manner. We conclude that growth of *A. thaliana* seedlings on high fungal doses of *P. indica* has little effect on the overall performance of the plants although elevated JA and JA-Ile levels in the roots induce a mild stress or defense response.

Introduction

Mutualism is a balanced stage of plant/microbe interaction where both partners benefit from each other.[1-3] In the symbiosis between the clavicipitaceous fungal endophyte *Epichloë festucae* and its host ryegrass, the degree of root colonization determines whether the interaction is mutualistic or parasitic.[4] In mycorrhiza, the beneficial interaction is based on the delivery of soil nutrients from the fungus to the plant and reduced carbon from the plant to the fungus.[5] However, environmental changes or mutations can shift these mutualistic interactions to commensalism (when one organism benefits without affecting the other) or parasitism (when one organism benefits while the other is harmed). Crucial for the maintenance of a mutualistic interaction is a balanced growth of the 2 symbionts, which requires a permanent signaling to establish an equilibrium between plant defense gene activation and propagation of the fungus.[1,2,6-13] This balance is also a prerequisite for appropriate reprogramming of the host development in response to endosymbionts.[14] The important role of defense gene activation in symbiotic interactions is shown for rice, where 43% of the genes respond to colonization by both arbuscular mycorrhiza fungi and pathogenic fungi, and many of them are involved in plant defense and stress.[15] Campos-Soriano and Segundo[8] proposed that increased demands for sugars by the fungus might activate host defense responses that will then contribute to the stabilization of root colonization. Plants may restrict carbohydrate flux toward their mycorrhizal partners to avoid fungal parasitism.[7] In tomato mycorrhiza, oxylipin metabolism and signaling may activate host defense responses that will contribute to both the control of fungal spread and the increased resistance to fungal pathogens in mycorrhizal plants.[9] Finally, Barto et al.[16] proposed fungal superhighways

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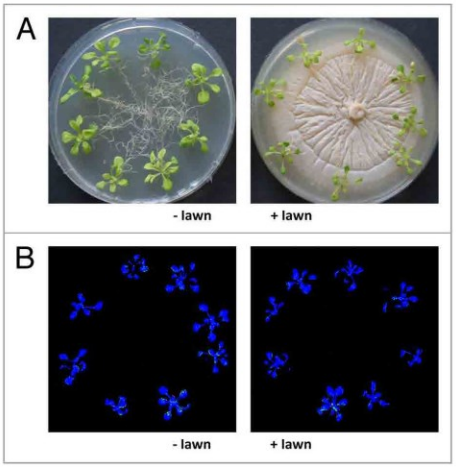

Figure 1. Plants exposed to a dense fungal lawn. (**A**) Nine-day-old Arabidiopsis wild-type seedlings were transferred from MS medium to a plate with KM without *P. indica* (left) or with a dense fungal lawn (right) for 7 d. Growth occurred in continuous light at 80 µmol min^{-2} sec^{-1}. (**B**) False color images of typical seedlings representing F_s/F_m values as described in Methods and Material and ref. 29.

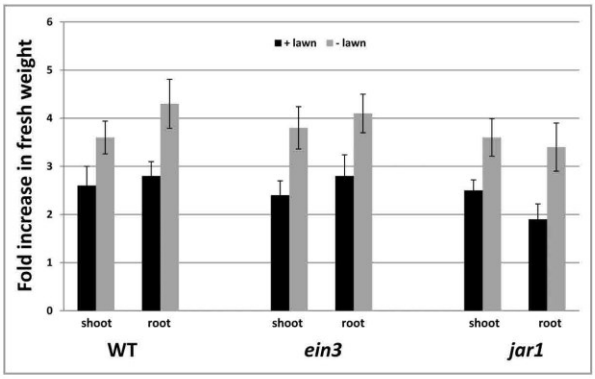

Figure 2. Fold increase in the fresh weight of the shoots and roots during the 7-d growth of the *Arabidopsis* wild-type (WT), *ein3*, and *jar1* seedlings on KM in the presence (+ lawn) or absence (- lawn) of *P. indica*. The values represent [fresh weight $_{9d\ MS\ +\ 7d\ KM\ \pm\ P.\ indica}$/fresh weight$_{9d\ MS}$]. Based on 6 independent experiments with 20 seedlings each, bars represent SEs. Errors are the sum of the individual errors.

that enhance belowground communication. In their model, infochemical transport via common mycorrhizal networks allows chemical defense signaling across plant populations. These examples demonstrate that regulation of plant defense and stress genes is crucial in symbiotic interactions and for the restriction of root colonization. A balanced activation of defense genes from the host is important to control fungal growth.

We study the interaction of *Arabidopsis* roots with the endophytic fungus *Piriformospora indica*, which colonizes the roots of many plant species. Similar to arbuscular mycorrhizal fungi, *P. indica* promotes plant growth, biomass, and seed production[17] and confers resistance to biotic and abiotic stress.[18,19] *P. indica* is a member of Sebacinales, grows inter- and intracellularly and forms pear-shaped spores, which accumulate within the roots and on the root surface.[20] After the establishment of a beneficial interaction, barely any defense or stress genes are activated and no reactive oxygen species (ROS) are produced by the host against *P. indica*.[3,21,22] Like in mycorrhizal symbiosis, the reason for the low level of plant defense against beneficial microbes in unknown. Jacobs et al.[23] proposed that *P. indica* is confronted with a functional plant immune system. It does not evade plant detection but rather suppresses immunity by various microbe-associated molecular patterns. Furthermore, they could show that the ability to suppress host immunity is compromised in the jasmonate mutants *jasmonate-insensitive 1* (*jin1*) and *jasmonate-resistant 1* (*jar1*). We and others have shown that the mutualistic interaction is disturbed in *Arabidopsis* mutants with lesions in specific defense genes or signaling processes leading to defense gene activation.[19,21,23-26] Mutants with lesions in a specific defense response are often unable to restrict growth of *P. indica* hyphae in the roots, and consequently, the roots become overcolonized. The host plant responds to it by activating other defense processes, which are not mutated in the host, to restrict fungal growth and to re-establish a balanced symbiosis of the 2 partners. In contrast to mycorrhizal fungi, *P. indica* can grow on synthetic media without a host.[27] Therefore we addressed the question of how *Arabidopsis* seedlings develop when they are growing on a dense fungal lawn. Do high fungal doses in the environment of the roots also lead to a higher colonization of the roots and, if so, does this affect plants' performance? Do the plants activate defense responses against the high fungal doses that surround the roots?

Results

High doses of *P. indica* inhibit growth but do not have any effect on the efficiency of the photosynthetic electron transport in *Arabidopsis* seedlings. When 9-d-old *Arabidopsis* seedlings were transferred from MS to Kaefer medium (KM) with or without a dense fungal lawn for 7 d (**Fig. 1A**), growth of the seedlings was slower on the medium with the fungus compared with the control. We observed a > 2-fold increase in the shoot and root fresh weights, respectively, for seedlings grown in the presence of the fungus for 7 d, while the increase on media without the fungus was ~3-fold (**Fig. 2**). The 2-fold increase in root and shoot fresh weight clearly indicates that the seedlings can grow on the fungal lawn. The slower growth rate in the presence of the fungus might be caused by an inhibitory effect of the fungus or simply by the fact that the access of the roots to nutrients in the agar medium is reduced (**Figs. 1A and 2**).

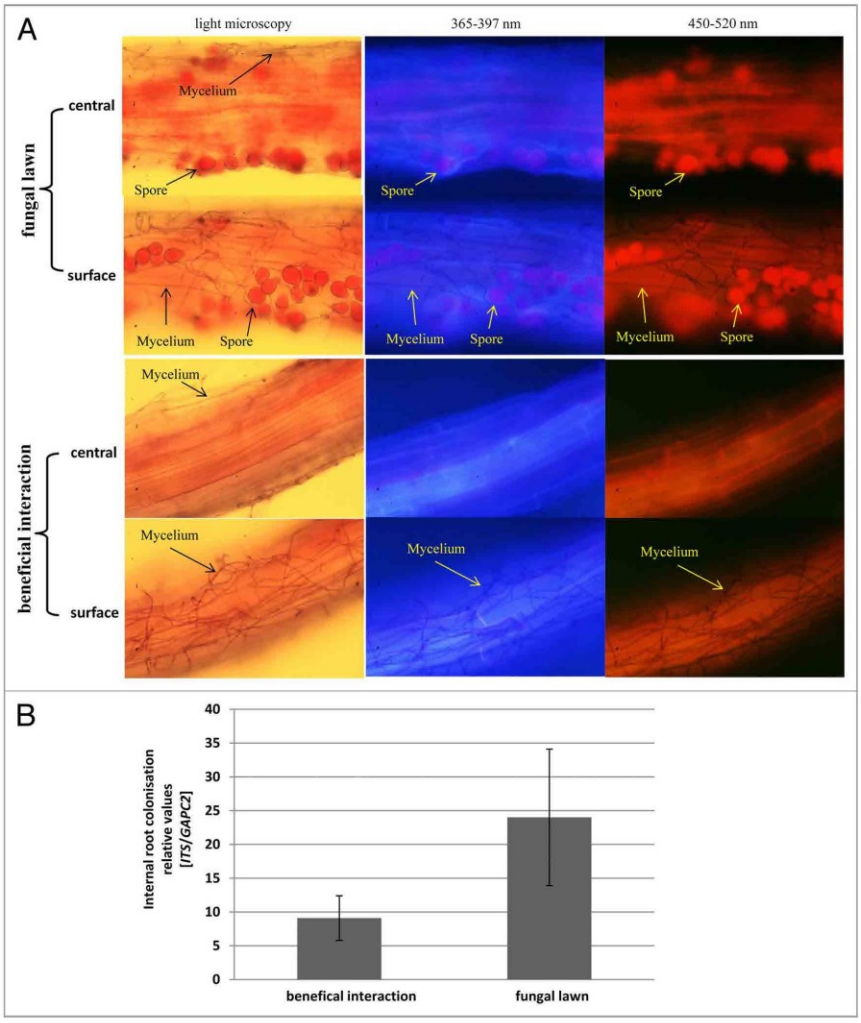

Figure 3. (**A**) Distribution of fungal mycelium and spores on the root surface and in the root material. The seedlings were either co-cultivated with *P. indica* on the fungal lawn for 7 d (upper part, fungal lawn), or co-cultivated with the fungus under beneficial conditions for 7 d (lower part, beneficial interaction). After staining of the fungal material, light microscopic or fluorescent pictures were taken from the root surface or from the central part of the root. Note the high concentration of spores and mycelium on the surface of the roots grown on the fungal lawn, which are not detectable inside of the roots. Under beneficial co-cultivation conditions for 7 d, spores are not yet formed, but the mycelium is detectable on the root surface. (**B**) The amount of the fungal *ITS* cDNA relative to the root *GAPC2* mRNA. *Arabidopsis* seedlings were co-cultivated with *P. indica* under beneficial conditions or on the fungal lawn for 7 d. After extensive washing of the roots, the mRNA was extracted and the amounts of the fungal and plant genes were determined by quantitative RT-PCR.

The efficiency of the photosynthetic electron flow, measured by chlorophyll fluorescence based parameters, is a sensitive parameter for the fitness of a plant.[28-30] After 7 d on KM either with or without the fungal lawn, the seedlings were dark-adapted for 15 min and the chlorophyll fluorescence was measured using a FluorCam 700F. False color images of the seedlings in plates (**Fig. 1A**) are shown in **Figure 1B** (compare ref. 29) and quantified data are presented in **Table 1**. The quantum yield of photosystem II Φ_{PSII}), photochemical (qP) and non-photochemical quenching (NPQ), and maximum quantum yield of PSII

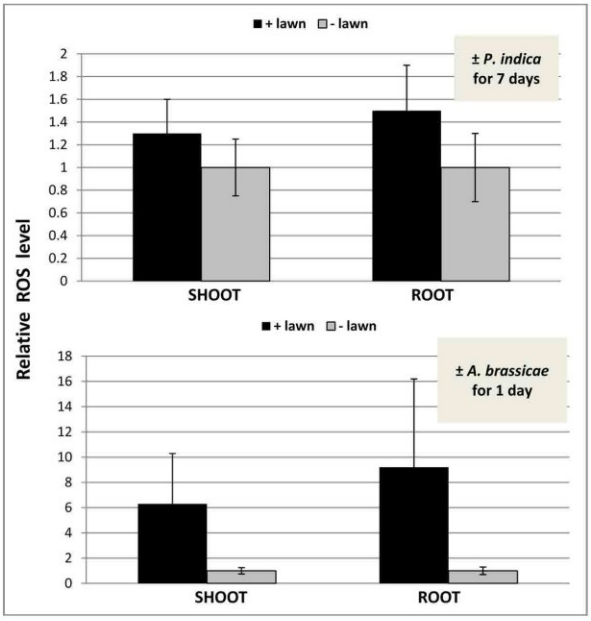

Figure 4. Relative ROS levels in the shoots and roots of *Arabidopsis* seedlings that were kept on KM medium with *P. indica* (+ lawn) or without *P. indica* (- lawn) for 7 d (top) or with *A. brassicae* (+ lawn) or without *A. brassicae* (- lawn) for 24 h (bottom). The ROS levels of the "– lawn" controls were set as 1.0 and the other values expressed relative to them. Based on 6 independent experiments with 20 seedlings each, bars represent SEs.

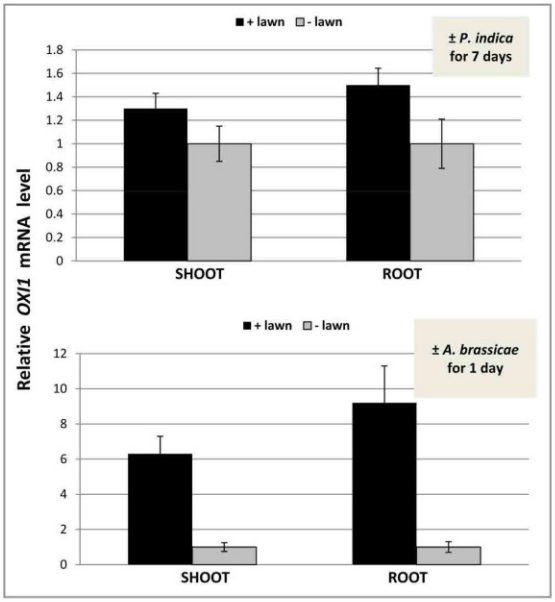

Figure 5. Relative *OXI1* mRNA level in the shoots and roots of *Arabidopsis* seedlings that were kept on KM medium with *P. indica* (+ lawn) or without *P. indica* (- lawn) for 7 d (top) or with *A. brassicae* (+ lawn) or without *A. brassicae* (- lawn) for 24 h (bottom). The *OXI1* mRNA levels in the "– lawn" samples were set as 1.0 and the other values expressed relative to them. Based on 6 independent experiments with 20 seedlings each, bars represent SEs.

(Fv/Fm) were almost identical for seedlings exposed to the dense fungal lawn and the control (**Fig. 1B**; **Table 1**). Thus, the efficiency of the photosynthetic electron transport (Φ_{PSII}, qP), the ability of heat dissipation of photochemical energy (NPQ), and the ratio of function photosystem II to total photosystem II (Fv/Fm) were not impaired by exposure of the seedlings to the dense fungal lawn. Furthermore, the amount of chlorophyll per gram fresh weight was not significantly different between the 2 treatments (**Table 1**). We have previously demonstrated that the efficiency of the photosynthetic electron transport is not affected or even slightly improved when the seedlings are exposed to a low concentration of the fungus and beneficial interaction conditions.[30] We conclude that the overall performance of the seedlings on the dense fungal lawn is quite well and comparable to seedlings that were not exposed to the dense fungal lawn, although their growth rate is reduced.

Intracellular growth of the mycelium on the dense fungal lawn is not significantly higher than under beneficial co-cultivation conditions

Growth of the seedlings on the dense fungal lawn results in a high concentration of mycelium and spores around the roots (**Fig. 3A**). After staining of the fungal material (compare Material and Methods) and microscopic analyses of the distribution of the mycelium and spores on the root surface and in the root material, we observed that the concentration of mycelium and spores inside the roots of seedlings grown on the fungal lawn was not higher than in roots that were grown with the fungus under beneficial co-cultivation conditions (**Fig. 3A**). This demonstrates that growth of the fungus inside the root material is independent of the concentration of the fungal material around the roots. This is further supported by quantitative RT-PCR analyses (**Fig. 3B**): the fungal *ITS* cDNA/plant *GAPC2* cDNA ratio is not significantly different in roots grown under beneficial co-cultivation conditions or on the fungal lawn.

High doses of P. indica do not stimulate H_2O_2/ROS production

Under beneficial co-cultivation conditions, *P. indica* does not induce H_2O_2/ROS production,[22] while exposure of roots to stress or pathogens is often associated with a massive ROS production. NBT staining of roots and shoots did not show any obvious difference between plant material exposed to the *P. indica* lawn and the mock-treatment (data not shown). Therefore, we used a more sensitive assay for quantitative measurement of ROS levels based on the Amplex Red

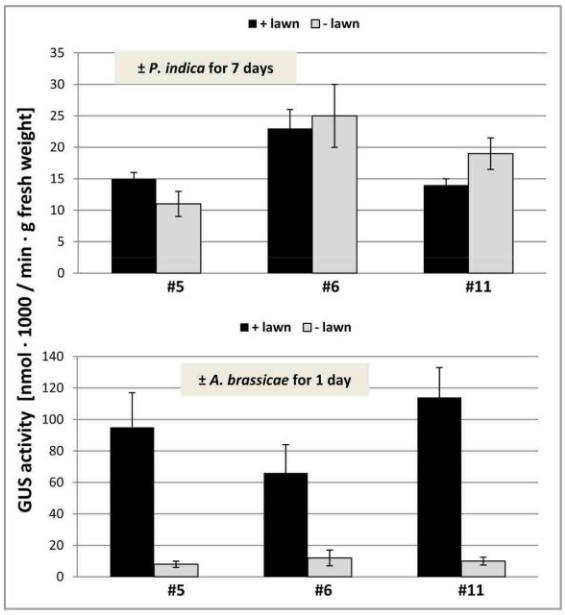

Figure 6. GUS activity in the roots of *Arabidopsis* seedlings that were kept on KM medium with *P. indica* (+ lawn) or without *P. indica* (- lawn) for 7 d (top) or with *A. brassicae* (+ lawn) or without *A. brassicae* (- lawn) for 24 h (bottom). The *uidA* gene was expressed under the control of the *oxi1* promoter (compare ref. 31). Three independent transformants (#5, #6, and #11) were analyzed. Based on 6 independent experiments with 20 seedlings each, bars represent SEs.

peroxidise technology (compare Methods and Material). Within 7 d, the overall ROS production in the roots of seedlings exposed to high fungal doses increased approximately 1.5–2-fold compared with seedlings grown in the absence of the fungus, but the difference was not significant (Fig. 4). The differences in the ROS levels in the leaves were even smaller than those in the roots (Fig. 4). We conclude that elevated ROS production cannot be observed even by high fungal doses. Furthermore, a ROS-inducible gene, *OXI1*,[31] is not significantly upregulated by *P. indica* in the shoots and roots (Fig. 5), and 3 independent *Arabidopsis* lines in which an *OXI1*::*uidA* construct was introduced[31] did not respond to *P. indica* (Fig. 6). For comparison, we performed the same cultivation experiment with *Arabidopsis* seedlings on a dense lawn of *Alternaria brassicae*. The measurements were performed after 24 h, since seedlings exposed to *A. brassicae* for 7 d were already dead (data not shown). A strong stimulation of ROS production can be measured within 24 h in the shoots and roots of the pathogen-exposed seedlings (Fig. 4). The *OXI1* mRNA level and expression of *OXI1*::*uidA* construct was strongly upregulated after 24 h (Figs. 5 and 6). Under these conditions, *OXI1* is induced by H_2O_2/ROS, which accumulate after pathogen attack.[31] The results indicate that exposure of *Arabidopsis* to high doses of *P. indica* over a longer period of time does not lead to the induction of substantial amounts of ROS in roots and shoots.

High doses of *P. indica* induce jasmonic acid and jasmonate-isoleucine levels in roots

Under beneficial co-cultivation conditions for 7 d, *P. indica* exposed and control seedlings of *A. thaliana* did not show differences in the jasmonic acid (JA) and jasmonate-isoleucine (JA-Ile) levels (unpublished). The JA and JA-Ile levels were > 2-fold upregulated in the roots of *Arabidopsis* seedlings grown on the dense fungal lawn for 7 d (Fig. 7). Interestingly, the stimulatory effect of the fungus was restricted to the roots and not observed for shoots of the same plant material, and the JA-Ile level in the shoots was even downregulated by the fungus (Fig. 7). This suggests a root-specific and not systemic effect of the high fungal doses on JA/JA-Ile levels. Furthermore, we included the *jar1* and *ein3* mutants into the study (compare below). Since JAR1 conjugates JA to Ile,[32-34] the JA-Ile levels are low in the roots and shoots of both *P. indica*-exposed and control *jar1* seedlings (Fig. 7). Finally, the JA level in roots of the *jar1* mutant was not upregulated on the dense fungal lawn. Therefore, upregulation of JA by *P. indica* in the roots requires JAR1 (Fig. 7).

Table 1. Chlorophyll concentration and photosynthetic electron transfer efficiency in the leaves of *Arabidopsis* seedlings grown on a dense fungal lawn of *P. indica*.

	Fungal Treatment	Chl (M Chl g-1 FW)	ΦPSII	qP	NPQ	Fv/Fm
WT	-	0,14 ± 0,02	0,83 ± 0,04	0,75 ± 0,05	0,33 ± 0,01	0,84 ± 0,02
WT	+	0,13 ± 0,04	0,87 ± 0,06	0,65 ± 0,07	0,34 ± 0,01	0,83 ± 0,01
jar1	-	0,13 ± 0,03	0,86 ± 0,05	0,73 ± 0,06	0,34 ± 0,01	0,83 ± 0,02
jar1	+	0,12 ± 0,04	0,83 ± 0,05	0,66 ± 0,05	0,35 ± 0,02	0,82 ± 0,03
ein3	-	0,13 ± 0,05	0,82 ± 0,07	0,77 ± 0,08	0,33 ± 0,01	0,84 ± 0,01
ein3	+	0,11 ± 0,06	0,89 ± 0,09	0,69 ± 0,09	0,36 ± 0,02	0,82 ± 0,02

Arabidopsis seedlings were kept on Kaefer medium either without (-) or with (+) the dense fungal lawn (compare **Figure 1A**) for 7 d and the chlorophyll content and fluorescence parameters were determined at the end of the experiment. The chlorophyll content per shoot fresh weight, the quantum yield of photosystem II (Φ_{PSII}), photochemical quenching (qP), non-photochemical quenching (NPQ), and the maximum yield of photosystem II (Fv/Fm) were measured for wild-type (WT), *jar1*, and *ein3* seedlings. Data are means ± SEs of 6 independent measurements with n = 5 (chlorophyll measurements) and n = 60 for the chlorophyll parameters.

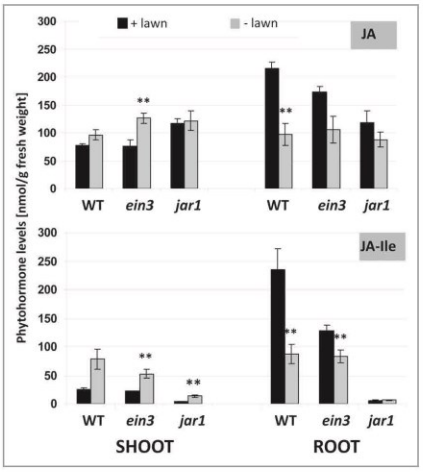

Figure 7. Phytohormone levels (jasmonic acid, JA; jasmonic acid isoleucine, JA-Ile) in the shoots and roots of wild type, *ein3*, and *jar1* seedlings. Nine-day-old seedlings were transferred from MS medium to a plate with KM without *P. indica* or with a dense fungal lawn for 7 d. The phytohormone levels for shoots and roots were analyzed separately. Based on 6 independent experiments with 20 seedlings each, bars represent SEs. **; p £ 0.01.

jar1 and _ein3_ seedlings do not suffer more than the wild-type from high fungal doses

To test whether JAR1 and EIN3 influence the performance of *Arabidopsis* seedlings on the dense fungal lawn, we compared wild-type, *jar1*, and *ein3* seedlings. However, we did not observe any visible differences in the growth behavior or fitness between the 3 genotypes (**Table 1**). The seedlings of all 3 genotypes grow slower in the presence of the fungus. Also, the chlorophyll content and the photosynthetic parameters were not different from the wild-type seedlings (**Table 1**). ROS production is not stimulated on the dense fungal lawn in the *jar1* and *ein3* mutants, again similar to the wild-type seedlings. We observed a < 2-fold stimulation of ROS production in the roots and shoots of 2 mutants, but these data were not significantly different from the untreated controls. Also the phenotype of their roots (root lengths and root architecture) did not differ when wild-type, *jar1*, and *ein3* seedlings were grown in the presence or absence of the fungus (data not shown). This suggests that JAR1 and EIN3 do not play an important role for the performance of *Arabidopsis* seedlings on a dense fungal lawn, although the JA and JA-Ile levels were increased in the roots.

High doses of _P. indica_ Strongly induce _PDF1.2_ and the Gene for a 2-oxoglutarate and Fe^{2+}-dependent oxygenase in the roots

PDF1.2 encodes a JA-inducible and ethylene (ET)-responsive plant defensin.[35] Consistent with the observation that the JA and JA-Ile levels were upregulated in the roots, we observed a > 10-fold upregulation of the *PDF1.2* mRNA level in *P. indica*-exposed wild-type roots compared with the mRNA level in roots that were not exposed to the fungus (**Fig. 8**). A ~7-fold stimulation of the *PDF1.2* mRNA level can be detected in the shoots (**Fig. 8**), although the JA and JA-Ile levels did not increase in the shoots on the dense fungal lawn. Therefore, the response in the shoots is systemic. The stimulatory effect in *jar1* and *ein3* seedlings was less than half compared with the wild-type, which indicates that JA/JA-Ile and also ET signaling is involved in *PDF1.2* expression. Interestingly, we identified another gene (*At4g10500*) encoding a 2-oxoglutarate and Fe^{2+}-dependent oxygenase that shows a similar regulation in the 3 genotypes (**Fig. 8**, cf. Discussion).

High doses of _P. indica_ induce only a mild upregulation of defense and stress genes

Next, we tested whether defense- and stress-related genes other than *PDF1.2* and *At4g10500* are upregulated on the fungal lawn, since accumulation of anthocyanin in the aerial parts and roots indicates that they are stressed to some extent (**Fig. 9**). We measured an approximately 6-fold upregulation of the anthocyanin level in the roots and 4-fold upregulation in the shoots of wild-type seedlings on the fungal lawn (**Fig. 9**). Comparable results were obtained for *ein3* and *jar1* seedlings (**Fig. 9**), which again confirms that the mutants do not suffer more than the wild-type when exposed to the high fungal doses. The elevated anthocyanin levels are also reflected by a ~2-fold stimulation of the mRNA for the *phenylalanine ammonium lyase* (*PAL*) *2* both in roots and shoots (**Table 2**) after 7 d on the fungal lawn. PAL2 is the main PAL isoform expressed in roots (compare Discussion). Neither the *PAL2* mRNA nor the anthocyanin levels are upregulated in roots or shoots under beneficial interaction conditions. For comparison, after 24 h on an *A. brassicae* lawn, a 22 ± 3-fold increase in the *PAL2* mRNA level was measured in the roots and a 6 ± 1-fold increase in the shoots (data not shown). Similar regulations in response to both *P. indica* for 7 d or to *A. brassicae* for 24 h were observed for the defense-related genes *PR2*, *PR3*, and *ERF1* in roots and shoots: the stimulatory effects by *P. indica* after 7 d were below a factor of 2.5 (**Table 2**), whereas those by *A. brassicae* after 24 h were > 9-fold in the roots and > 5-fold increase in the shoots (data not shown). Again, the expression levels of *PAL2*, *PR2*, *PR3*, and *ERF1* in *P. indica*-exposed or mock-treated *ein3* and *jar1* roots were comparable to those in the wild-type (based on 6 independent experiments with 20 plants each). Taken together, quite different defense and stress genes are only mildly upregulated in the roots and shoots of *Arabidopsis* seedlings even exposed to high doses of *P. indica* for 7 d.

To test whether other genes with stress- and defense-related functions are upregulated in *Arabidopsis* roots exposed to the fungal lawn of *P. indica*, we tested genes that were not upregulated under beneficial co-cultivation conditions of the 2 symbionts, but that responded > 10-fold to unbalances in the symbiontic interaction (Vahabi et al., manuscript in preparation). Interestingly, only *At4g10500* (**Fig. 8**) showed a strong response to the fungal lawn, while all other genes were upregulated less than 2.5-fold (**Table 2**) and thus comparable to *PAL2*, *PR2*, *PR3*, and *ERF1*. This includes genes for P450 enzymes, the calmodulin-binding protein CBP60 g, a chitinase, the stress-related RmlC-like cupins protein At5g38910, the trypsin inhibitor ATT1, a

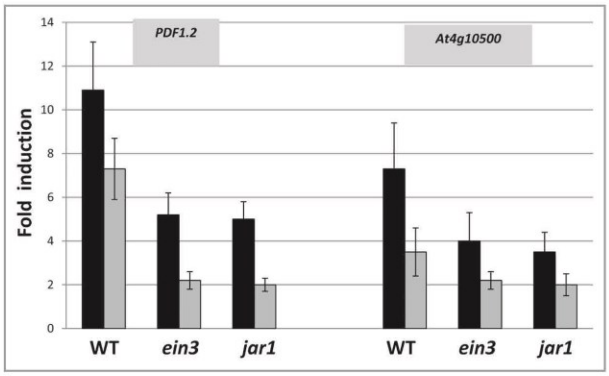

Figure 8. Fold-induction of *PDF1.2* and *At4g10500* transcripts levels in the roots (black) and shoots (gray) of wild-type (WT), *ein3*, or *jar1* seedlings that were either kept on the fungal lawn of *P. indica* or mock-treated. Nine-day-old seedlings were transferred from MS medium to a plate with KM without or with a fungal lawn for 7 d before the RNA was extracted from their roots and shoots. The values indicate fold induction ($mRNA_{+P.\ indica}/mRNA_{-P.\ indica}$). Based on 6 independent experiments with 20 seedlings each, bars represent SEs. Errors are the sum of the individual errors.

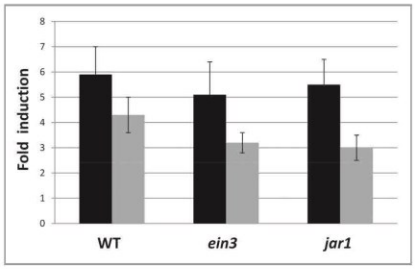

Figure 9. Fold-induction of anthocyanin levels in the roots (black) and shoots (gray) of wild-type (WT), *ein3* and *jar1* seedlings that were either kept on the fungal lawn of *P. indica* or mock-treated. Nine-day-old seedlings were transferred from MS medium to a plate with KM without or with a fungal lawn for 7 d before the anthocyanin level was determined for the roots and shoots. The values indicate fold induction ($A_{530+P.\ indica}/A_{530-P.\ indica}$). For experimental details cf. Material and Methods. Based on 9 independent experiments with 20 seedlings each, bars represent SEs. Errors are the sum of the individual errors.

glutathione-S-transferase (At1g02930), and the GDSL-lipase GLIP1. Several of the genes are involved in JA (CBP60 g, CYP82C2), ABA (CBP60 g), cytokinin (2-oxoglutarate and Fe^{2+}-dependent oxygenase), and ET (GLIP1) signaling (compare Discussion). We conclude that growth on the fungal lawn does not induce a massive stress response in the seedlings (compare Discussion).

Discussion

The overall observation is that growth of *Arabidopsis* seedlings on a dense fungal lawn does not result in a higher fungal concentration in the root material. The performance of the plants, measured on the basis of the efficiency of the photosynthetic electron flow, is not impaired on the dense fungal lawn, compared with plants grown without the fungus or under beneficial co-cultivation conditions. We also did not observe a massive defense or stress response in roots or shoots, although the growth rate of the seedlings is reduced compared with control seedlings grown on the KM without the fungus (**Figs. 1A and 2**). The reduced growth rate may be caused by inhibitory factors from the fungus or by the fact that the seedlings do not have direct access to essential nutrients in the agar. Since accumulation of chlorophyll per gram leaf tissue and the efficiency of the photosynthetic electron transport is not or barely impaired on the fungal lawn (**Table 1**), the plants are fit and do not suffer. We cannot detect significant increases in H_2O_2/ROS production (**Fig. 4**), the stimulation of the H_2O_2 inducible *OXI1* gene (**Fig. 5**), and the activation of the H_2O_2-inducible *oxi1* promoter (**Fig. 6**) in the *P. indica*-exposed tissue, which is consistent with previous observations.[22,36] Furthermore, classical defense genes such as *PR2* (encodes a β-1,3-glucanase), *PR3* (encodes a basic chitinase), and *ERF1* (encodes an ET responsive element binding factor) are only mildly upregulated (**Table 2**). ERF1 has been included in this study because it has previously been demonstrated that this transcription factor gene is involved in the *Arabidopsis*/*P. indica* interaction.[24,25] The elevated anthocyanin and *PAL2* mRNA levels in the *P. indica*-exposed seedlings (**Fig. 9**; **Table 2**) suggest that the seedlings suffer to some extent, although this does not have an effect on the efficiency of the photosynthetic electron transport in the leaves (**Table 1**). Four *PAL* genes are present in the *Arabidopsis* genome,[37] and they respond to a multitude of environmental stress stimuli including pathogen infection, wounding, nutrient depletion, UV irradiation, or extreme temperatures.[38-40] *PAL2* is mainly expressed in roots, and we could show that this gene is only marginally upregulated on the *P. indica* lawn (**Table 2**), when compared with the induction by *A. brassicae*. Furthermore, growth of wild-type, *jar1*, and *ein3* seedlings on the dense fungal lawn was reduced compared with seedlings that were grown without the fungus, but the reduction was similar for the 3 genotypes, and the performance of the seedlings were identical (**Table 1**). Since also stimulation of anthocyanin, and of the *PAL2*, *PR2*, *PR3*, and *ERF1* mRNA levels were comparable for the 3 genotypes (**Table 2**), neither EIN3 nor JAR1 have a significant influence on the performance of the seedlings on the fungal lawn. The lack of massive defense gene activation in the roots exposed to a dense fungal lawn suggests that *P. indica* is either unable to release microbe-associated molecular patterns that activate defense processes, or the fungus activates mechanisms to repress their activation. Jacobs et al.[23] suggested that *P. indica* has established efficient mechanism(s) to bypass or supress host immunity, since the fungus is confronted with a functional root immune system. Either this is also the case for

Table 2. Stress- and defense-related genes and their regulation in the roots (and shoots) after growth of wild-type (WT), *ein3*, or *jar1* seedlings on a dense fungal lawn of *P. indica* for 7 d (**Fig. 1A**)

WT seedlings			
Acc. No.	**Protein**	**Fold stimulation in roots**	**Fold stimulation in shoots**
At3g53260	PAL2	1,6 ± 0,4	1,2 ± 0,3
At3g57260	PR2	1,9 ± 0,4	1,4 ± 0,5
At3g12500	PR3	1,3 ± 0,4	1,5 ± 0,5
At4g17500	ERF1	1,5 ± 0,3	1,2 ± 0,4
***ein3* seedlings**			
Acc. No.	**Protein**	**Fold stimulation in roots**	**Fold stimulation in shoots**
At3g53260	PAL2	1,4 ± 0,4	1,4 ± 0,4
At3g57260	PR2	1,5 ± 0,3	1,5 ± 0,5
At3g12500	PR3	1,5 ± 0,4	1,5 ± 0,7
At4g17500	ERF1	1,2 ± 0,4	1,7 ± 0,3
***jar1* seedlings**			
Acc. No.	**Protein**	**Fold stimulation in roots**	**Fold stimulation in shoots**
At3g53260	PAL2	1,2 ± 0,5	1,7 ± 0,5
At3g57260	PR2	1,8 ± 0,3	1,6 ± 0,4
At3g12500	PR3	1,6 ± 0,5	1,9 ± 0,7
At4g17500	ERF1	1,7 ± 0,4	1,8 ± 0,5
WT seedlings			
Acc. No.	**Protein**	**Fold stimulation in roots**	**References**
At5g26920	Calmodulin-binding protein CBP60 g	1,3 ± 0,3	51–54
At4g31970	P450 enzyme CYP82C2	1,1 ± 0,3	55, 56
At5g38910	RmlC-like cupins protein	1,5 ± 0,4	58 57
At5g25260	Plasmamembrane protein	1,8 ± 0,4	59, 60
At4g11170	Disease response protein	1,3 ± 0,4	61
At3g60270	Cupredoxin protein	1,1 ± 0,2	
At5g57220	P450 enzyme CYP81F2	2,2 ± 0,9	62
At5g40990	GDSL lipase 1	1,5 ± 0,3	63
At1g26390	Berberine protein	1,4 ± 0,4	64–66
At5g39580	Peroxidase	1.6 ± 0,2	67
At2g30750	P450 enzyme CYP1A12	1,3 ± 0,3	68

The values show fold induction relative to the mRNA levels in the roots/shoots of seedlings that were grown on KM medium without the fungus. Based on 3 independent real-time PCR analyses, errors represent SEs. The errors represent the sum of the 2 errors of the original data.

our co-cultivation conditions, or the 2 co-cultivation conditions cannot be compared.

The results shown here open the question why the cultivation of *A. thaliana* on a dense fungal layer does not result in a massive infection (**Fig. 3**). The roots may either have established mechanisms to control fungal invasion for their protection against overcolonization, or intracellular root colonization follows a highly regulated program that is independent of the fungal concentration around the roots and in the rhizosphere. The absence of a massive defense response of the roots against *P. indica* is consistent with the colonization data (**Fig. 3**). For mycorrhizal interaction, empirical studies have shown that mycorrhizal colonization intensity exhibits substantial heritable genetic variation within plant and fungal species and are influenced by plant genotype/fungal genotype interactions, suggesting the potential for ongoing coevolutionary selection.[41] It might be possible that plant genetic traits limit root colonization in the *P. indica*/*Arabidopsis* symbiosis. It has also been discussed

that root colonization maybe controlled by a phosphate/carbon exchange between the 2 partners, and that limitations in any of the nutrients may limit the colonization process.[42] In mycorrhizal interactions, exchange of small molecules from both partners may determine the degree of root colonization.[43] Candidates for small molecules from the fungus that may be important for the control of root colonization are discussed, after the entire *P. indica* genome has been sequenced.[44,45] Finally, JA and salicylic acid signaling during specific stages of root colonization may control a balance between compatibility and defense in mutualistic interactions,[46] and this might be also the reason why the level of the active form of JA increases on the dense fungal lawn. Since the *jar1* mutant does not suffer on the lawn during the 7 d of co-cultivation, it appears that this phytohormone is not required during early phases of the interaction. However, the phytohormone might be required during later phases of the interaction. Finally, Lahmann and Zuccaro[47] and others describe a biphasic colonization strategy of barley and *Arabidopsis* roots by *P. indica* upon penetration of the root: Perturbance of plant hormone homeostasis and secretion of fungal lectins and other small proteins (effectors) may be involved in the evasion and suppression of host defenses at these early colonization steps. At later stages, *P. indica* is found more often in moribund host cells where it secretes hydrolytic enzymes. This strategy of colonizing plants is reminiscent of that of hemibiotrophic fungi, although a defined shift to necrotrophy with massive host cell death is missing. It is reasonable to assume that the plant has established mechanisms that specifically counteract cell-death inducing processes by restricting fungal invasion or growth inside the plant tissue, in particular if the symbiosis is beneficial for the plant.

We identified only 2 genes that were strongly upregulated in response to the fungal lawn. One of these genes is *PDF1.2* (**Fig. 8**). The response is consistent with elevated JA and JA-Ile levels (**Fig. 7**). *PDF1.2* is a marker gene for JA and ET signaling.[35] Since the *PDF1.2* mRNA level is less upregulated in *jar1* and *ein3* roots compared with wild-type roots (**Fig. 8**), it is likely that both phytohormones are involved in the induction. *PDF1.2* is induced both locally at the site of infection by incompatible fungal pathogens and systemically in remote noninoculated regions of the plant.[48] This activation occurs via the JA/ET rather than the SA pathway.[35,48] Necrotrophic fungi can inhibit JA-induced defense gene activation, as recently shown for SSITL, an effector from *Sclerotinia sclerotiorum* that plays a significant role in the suppression of JA/ET signal pathway-mediated resistance at the early stage of infection.[49] Interestingly, *PDF1.2* was also upregulated in the leaves of *P. indica*-exposed seedlings (**Fig. 8**), suggesting a systemic effect, although the JA/JA-Ile levels were not higher in the leaves (**Fig. 7**). Suza et al.[34] have shown that *jar1* has little or no impact on several wound-induced genes. To test for a possible JAR1 role in systemic induction, younger unwounded leaves from the same wounded plants were examined. *PDF1.2* transcripts accumulated in both wounded and unwounded leaves, and *jar1* did not affect the timing or magnitude of accumulation. This provides an example for a JAR1-independent regulation of *PDF1.2*, and might be comparable to the upregulation of *PDF1.2* in leaves where the JA/JA-Ile levels were not elevated, as well as to the upregulation of *PDF1.2* in *jar1* plants on the fungal lawn (**Figs. 7** and **8**). Finally, *ein3* usually shows a lower *PDF1.2* expression compared with the wild type,[50] consistent with our observations (**Fig. 8**).

Whether *PDF1.2* regulation is a defense response or a general stress response under our co-cultivation conditions is unclear. In barley *P. indica* elicits a non-specific defense reaction by upregulation of a multiplicity of stress responsive genes.[51] If this is also true for the interaction studies described here, the fungus induce only a mild stress response in *Arabidopsis* roots, which has little effect on plant performance and no effect on the efficiency of the photosynthetic electron transport (**Table 1**). Interestingly, the 2-oxoglutarate and Fe^{2+}-dependent oxygenase-encoding gene *At4g10500* shows the same regulation as *PDF1.2* on the fungal lawn (**Fig. 8**). The gene is stimulated in response to calcium stress,[52] senescence,[53] and is involved in cytokinin signaling.[54] The message is also upregulated in *powdery mildew resistant 4* (*pmr4*), a mutant lacking pathogen-induced callose.[55] Further studies are required to understand the role of this protein in the symbiotic interaction.

We also analyzed the expression of a set of less studied defense- and stress-related genes, but all of them were barely or not induced in the roots in response to the dense fungal lawn (**Table 2**). ERF1, a target transcription factor of both JA and ET signaling, is important for *PDF1.2* activation[56,57] and involved in the *Arabidopsis*/*P. indica* interaction.[24,25] CBP60 g is a calmodulin-binding protein that has previously been described to respond to *P. indica* under beneficial co-cultivation conditions in wild-type roots.[58] The protein is a positive regulator of both disease resistance and drought tolerance in *Arabidopsis*.[59] Overexpression of CBP60 g caused elevated SA accumulation, increased expression of the defense genes, enhanced resistance to *Pseudomonas syringae*, hypersensitivity to abscisic acid (ABA), and enhanced tolerance to drought stress.[59] CBP60 g has a partially redundant role with SAD1, which affect defense responses in addition to SA production.[60,61] The P450 protein CYP82C2 (At4g31970) modulates JA-induced root growth inhibition, defense gene expression, and indole glucosinolate biosynthesis.[62] CYP82C2 affects JA-induced accumulation of tryptophan, but not the JA-induced auxin- or pathogen-induced camalexin, and thus acts in the metabolism of tryptophan-derived secondary metabolites under conditions in which JA levels are elevated.[62] The enzyme is also involved in the systemic resistance response induced by the root-colonizing *Pseudomonas fluorescens* strain SS101 against several bacterial pathogens, including *Pseudomonas syringae* pv tomato, and the insect pest *Spodoptera exigua*.[63] The RmlC-like cupins protein At5g38910 is an apoplastic manganese ion binding protein with potential nutrient reservoir activity. The mRNA was induced by cesium stress[64] as well as treatments with 9 other abiotic stresses.[65] The mRNA for the plasma membrane-associated protein At5g25260 is induced in response to geminivirus[66] and *Pseudomonas syringae*[67] infections, and involved in ABA-mediated defense responses.[67] At4g11170 is a defense protein, which responds to ozone and shows a high expression in stems, roots, and stamen.[68] The failure of this gene to respond to the dense fungal lawn is

consistent with the observation that the H_2O_2/ROS levels do not increase. The P450 enzyme CYP81F2 (At5g57220) is involved in glucosinolate biosynthesis and *Arabidopsis* innate immune responses.[69] The GDSL lipase-like 1 (At5g40990) regulates systemic resistance associated with ET signaling[70] and elicits both local and systemic resistance. GLIP1-overexpressors exhibited enhanced resistance against necrotrophic pathogens, including *A. brassicicola* and *Erwinia carotovora*, and the hemibiotrophic pathogen *Pseudomonas syringae*.[70] The FAD-binding berberine protein At1g26390 is an oxidoreductase in the endomembrane system that is upregulated in response to various biotic and abiotic stresses.[71-73] The identified peroxidase At5g39580 is involved in various defense responses.[74] Finally, the P450 enzyme CYP1A12 is involved in camalexin synthesis and thus crucial for defense responses against pathogens and herbivores.[75] All these genes have been identified in previous studies to be upregulated > 10-fold when the symbiotic interaction between *P. indica* and *Arabidopsis* is disturbed (Vahabi et al., unpublished). The lack of regulation of these genes on the dense fungal lawn suggests that the interaction of the 2 symbionts is not harmful. The exact function of these proteins in the *P. indica*/*Arabidopsis* symbiosis under unfavorable co-cultivation conditions is currently under study.

Under beneficial co-cultivation conditions, defense and stress genes including *PDF1.2* are not upregulated in *Arabidopsis* roots.[3,18,19,21,22,24-26] However, *Arabidopsis* mutants, which are impaired in establishing a beneficial interaction or are unable to maintain a long-term harmony between the 2 symbionts, activate a mild defense response against *P. indica*.[18,19,21,22,24-26] In particular, *PDF1.2* has been reported repeatedly as being upregulated under these conditions.[19] Unbalances in the interaction are often accompanied by an increase in root colonization.[19] This has also been observed for other mutualistic interactions, e.g., for mycorrhiza formation[4,46,76,77] and the interaction of plant-growth promoting microbes with roots.[78,79] The results shown here are somewhat surprising since they show that *Arabidopsis* seedlings can be exposed to a high dosis of a beneficial fungus without a significant defense gene activation from the host against the microbe or shift from mutualism to parasitism. The plants perform quite well. The available tools and genes described here allow us to investigate the signaling between the symbionts in greater details. In particular, the results demonstrate that these genes are not simply upregulated if the roots are surrounded by high fungal doses of *P. indica*.

Material and Methods

Growth conditions of plants and fungus

Wild-type or mutant (*jar1*, obtained from Dr. J. Vadassery, Max-Planck-Institute for Chemical Ecology and *ein3*[24]) *Arabidopsis thaliana* seeds were surface-sterilized and placed on petri dishes containing MS nutrient medium.[80] After cold treatment at 4 °C for 48 h, plates were incubated for 7 d at 22 °C under continuous illumination (75 µmol m^{-2} sec^{-1}). *P. indica* was cultured as described previously[20,81,82] on KM modified by Pham et al.[82] For solid medium 1% (w/v) agar was included. *Alternaria brassicae* cultivation has been described in details in Johnson et al.[83]

Nine-day-old wild-type, *ein3*, or *jar1* seedlings were directly transferred from MS medium to a plate with a fungal lawn of *P. indica*.[83] The fungal lawn was obtained by placing a fungal plug on KM and the fungus was allowed to grow for 14 d at 24 °C in the dark, before the seedlings were transferred to the plate. Control seedlings were transferred to KM without the fungus. The plates were incubated for 7 d at 22 °C under continuous illumination (µmol m^{-2} sec^{-1}) from above.[83] The co-cultivation experiments with *A. brassicae* were performed under the same conditions, except that the fungus colonized the plate only for 5 d[83] and co-cultivation was terminated after 24 h. Fresh weights were determined directly after harvest. Beneficial co-cultivation conditions of the 2 symbionts for 7 d was performed on PNM medium, because growth of the fungus on Kaefer medium is too fast for a balanced interaction of *P. indica* and *A. thaliana*.[20] A detailed protocol is given in Johnson et al.[83]

Staining of fungal mycelium and spores

Fuchsin acid and trypan blue staining of fungal hyphae in *Arabidopsis* roots was described in details in Vahabi et al.[84] In brief, *Arabidopsis* roots co-cultivated with *P. indica* were collected and intensively washed with distilled water. After incubation in fuchsin acid solution for 10 min, the material was washed with destilled water for 1 min. They were then stained with trypan blue (0.0001mg/ml) solution for 3 min and washed again for 1 min.

After addition of 50 µl GL solution (glycerol:lactic acid:water, 1:1:1) on a glass slide, the sample was protected with a glass cover, before analysis by light and fluorescent microscopy using different wavelengths. The localization of hyphae and spores on the surface of the roots or in the root material was distinguished by scanning through the plant material.

RNA extraction and cDNA synthesis

RNA was isolated from the roots or shoots with an RNA isolation kit (RNeasy, Qiagen, Hilden, Germany). For quantitative RT-PCR, RNA from *Arabidopsis* mock-treated roots/shoots and roots/shoots treated with a high amount of fungal hyphae were used. Reverse transcription of 1 µg of total RNA was performed with an oligodT Primer. First strand synthesis was performed with a kit from Qiagen (Omniscript RT Kit, Qiagen, Hilden, Germany).

Real-time PCR

Real-time quantitative RT-PCR was performed using the iCycler iQ real time PCR detection system and iCycler software version 2.2 (Bio-Rad, Munich, Germany). For the amplification of the PCR products, iQ SYBR Supermix from Bio-Rad was used according to the manufacturer's instructions in a final volume of 23 µl. The iCycler was programmed to 95 °C 3 min, 40 × (94 °C 30 s, 57 °C 30 s, 72 °C 40 s), 72 °C 10 min followed by a melting curve program (50–85 °C in increasing steps of 0.5 °C). All reactions were repeated at least 4 times. The mRNA levels for each cDNA probe were normalized with respect to the *GAPC2* message level. Fold induction values were calculated with the ΔΔCP equation of Pfaffl.[85] *P. indica* cDNA was detected with primers for the *ITS* region (CAACACATGTGCACGTCGAT;

Box 1. The following *Arabidopsis* primer pairs were used:

Gene	Forward primer	Reverse primer
At5g26920 (CBP60G, CaM-binding protein)	CCGCATTACA GCGGTTAACG ATAG	ACTTCCTTGA AAGTCGATGT GCTG
At5g38910 (RmlC-like cupins superfamily protein)	TATTGCTGAC ACCGTGTTTG GG	ACTTCCTTGA AAGTCGATGT GCTG
At5g39580 (Peroxidase superfamily protein)	GCGATCTCGT CACTCTTGTT GGAG	TAAACCCACA TGCAGCTGTT CCG
At5g40990 (GDSL lipase1)	CCTGATTTCA TCGCGGAGTA CG	TGGCTGTTACC GTTGAATGGT TG
At5g57220 (CYP81F2, cytochrome P450)	TCATCAAAGG GCTCATGCTC AG	GCCATCGCCC ATTCCAATGT TAC
At1g26390 (FAD-binding berberine family protein)	ACGCCACAAT GAGTAGCCTG AG	TCACCACTCG GATTGCTTCC AAC
At3g60270 (Cupredoxin superfamily protein)	TGCAGCCTTG GCATGAAACT CG	ACGGTGGAGG CTCTAATGAA ACG
At5g25260 (PHB domain-containing membrane-associated protein family)	TTGCTAAGAC TAACGCGCTT GC	GTTCTCCACC ATGGTTCCAA ACG
At4g31970 (CYP82C2, cytochrome P450)	ATTAAATCTA CCTGCCTGGC ACTG	GCCCATGTAA GGGTTGATGG TG
At4g11170 (TIR-NBS-LRR class)	AGAAGCTATG GAGTGGAGTT CAGC	AGCTCCACCA AAGACTCACA CC
At5g44420 (PDF1.2)	CTTGTGTGCT GGGAAGACAT A	AGCACAGAAG TTGTGCGAGA A
At4g10500 (2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein)	TATCGGCGAC CAAATGCAGG TC	ACTACGGCTC TATGGAGCAC AC
At3g57260 (PR2)	TCTTCTCAGC CTTGTAATAG C	TGTTTGTAAA GAGCCACAAC G
At3g12500 (PR3)	TCATGGGGCT ACTGTTTCAA G	TATTGCTCTA CCGCATAGAC C
At4g17500 (ERF1)	TATCCTCAAC GACGCCTTTC	TCTTGACCGG AACAGAATCC
At3g53260 (PAL2)	AGGTACTGAC AGTTACGGAG	CATGTCTCCT TCGTGTTTCC
At3g04120 (GAPC2)	GAGCTGACTA CGTTGTTGAG	GGAGACAATG TCAAGGTCGG

CCAATGTGCA TTCAGAACGA). Root colonization was determined relative to the plant *GAPC2* cDNA levels (See **Box 1**).

Phytohormone measurement

Phytohormones were extracted by homogenizing approximately 100 mg of *Arabidopsis* material and adding 1 ml ethylacetate spiked with internal standards [D6-ABA, D2-JA, D4- SA, and 13C6-JA-Ile (200 ng/each)]. Samples were homogenized twice by reciprocal shaking (FastPrep speed 6.5) for 45 s and centrifuged at 13 000 rpm for 20 min at 4 °C. The supernatant was transferred to a fresh 2 ml microcentrifuge tube and the extraction was repeated by adding 1 ml ethylacetate without internal standards. The organic phases were pooled and evaporated under reduced pressure. The dried sample was dissolved in 500 µl 70% methanol, vortexed, and centrifuged at 13 000 rpm for 10 min. 100 µl of the supernatant were transferred into an HPLC vial with insert and the samples were analyzed by liquid chromatography tandem mass spectormetry system (Varian 1200L Triple-Quadrupol-MS). Ten µl of each sample were injected onto a ProntoSIL column (C18; 5µm, 50 × 2mm). The mobile phase comprised solvent A (0.05% formic acid in water) and solvent B (0.05% formic acid in acetonitrile). Compounds were ionized by electrospray ionization and analyzed in the negative mode by multiple reaction monitoring (MRM).

NBT stain

Arabidopsis seedlings were grown as described before. After 7 d on the fungal lawn, the roots and shoots were stained for 5 min in a solution containing 2 mM nitrobluetetrazolium (NBT; Sigma Aldrich) in water. The reaction was stopped by washing the roots with water. Roots were evaluated under the Axiovert 135 (Carl Zeiss MicroImaging GmbH, Jena, Germany).

ROS measurements

Quantitative ROS measurements from leaves and roots were performed using the Amplex Red hydrogenperoxide/peroxidase assay kit (Molecular Probes) according to the manufacturer's instructions (http://tools.invitrogen.com/content/sfs/manuals/mp 22188.pdf) using the substrate carboxy-H_2DFFDA (Molecular Probes) according to the manufacturer's instructions (https://tools.invitrogen.com/content/sfs/manuals/mp36103.pdf). Leaf sections of 0.5–1 mm width and root sections of 2–3 cm length were incubated in 20 µM carboxy-H_2DFFDA prepared in KRPG buffer for 30 min in the dark. The fluorescence intensity was quantified with a fluorescence microplate reader (TECAN Infinite 200) with an excitation at 485 nm and emission at 530 nm. The reaction mixture without the substrate and plant material served as control.

Anthocyanin measurements

Roots and shoots were ground in liquid nitrogen and extracted with 80% methanol/5% HCl in the dark and incubated overnight at 4 °C. After centrifugation (15.000 g, 20 min) the supernatant was removed and the anthocyanin concentration was determined spectrophotometrically with a Lambda 12 spectrophotometer (Perkin-Elmer Company). The amount of anthocyanin is expressed as A_{530}/mg fresh weight.

GUS assay

Oxi promoter::*uidA* lines[31] were grown on MS media with the appropriate amount of kanamycin for 9 d and were then exposed for 5 d to the treatments. Whole plants were harvested and ground with 500 µl of lysis buffer (100 mM Na_3PO_4, pH 7.0; 500 mM EDTA; 0,1% Triton X-100; 0,1% lauroyl sarcosin; 10 mM β-mercaptoethanol). The homogenate was clarified

by centrifugation and 10 µl of the supernatant was used for assaying GUS activity in a total volume of 100 µl with 1 mM methylumbelliferyl-β-D-glucoside in lysis buffer. The enzyme reaction was performed at 37 °C and stopped after 30 min with 900 µl 100 mM Na_2CO_3. Samples were measured with the fluorometer VersoFluor from Bio-Rad (Bio-Rad, Munich, Germany) after setting the range according to the manufacturer's instructions. The system of reference was an equal amount of fresh weight.

Measurement of chlorophyll and photosynthesis parameters

Measurement of chlorophyll content was performed as described in Porra et al.[86] The *Arabidopsis* seedlings were dark-adapted for 15 min and then the chlorophyll fluorescence was measured using a FluorCam 700F (Photon System Instruments, Czech Republic). Program parameters of FluorCam were set according to Wagner et al.[29] Photosynthesis parameters, quantum yield of PSII (Φ_{PSII}), maximum quantum yield of PSII (Fv/Fm), photochemical quenching (qP), and non-photochemical quenching (NPQ) were calculated according to Maxwell and Johnson.[28] False color images of the seedlings in plates were obtained as described by Wagner et al.[29] Chlorophyll fluorescence images representing F_s/F_m values are shown, whereas blue represents low F_s/F_m values above a threshold of 0.06 and red represents high F_s/F_m values with an upper threshold limit of 0.17.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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4.2 Manuscript II

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The interaction of Arabidopsis with *Piriformospora indica* shifts from initial transient stress induced by fungus-released chemical mediators to a mutualistic interaction after physical contact of the two symbionts

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Abstract

Background: *Piriformospora indica*, an endophytic fungus of Sebacinales, colonizes the roots of many plant species including *Arabidopsis thaliana*. The symbiotic interaction promotes plant performance, growth and resistance/tolerance against abiotic and biotic stress.

Results: We demonstrate that exudated compounds from the fungus activate stress and defense responses in the Arabidopsis roots and shoots before the two partners are in physical contact. They induce stomata closure, stimulate reactive oxygen species (ROS) production, stress-related phytohormone accumulation and activate defense and stress genes in the roots and/or shoots. Once a physical contact is established, the stomata re-open, ROS and phytohormone levels decline, and the number and expression level of defense/stress-related genes decreases.

Conclusions: We propose that exudated compounds from *P. indica* induce stress and defense responses in the host. Root colonization results in the down-regulation of defense responses and the activation of genes involved in promoting plant growth, metabolism and performance.

Keywords: Microarray, Transcriptome, Defense, Mutualism, Stomata, Reactive oxygen species, Phytohormones

Background

The mutualistic interaction between beneficial root-colonizing fungi or bacteria starts with the recognition of both partners before a physical contact is established. Mutual recognition of diffusible signals released by the roots and microbes [arbuscular mycorrhizal (AM), rhizobia-legume root endosymbionts, beneficial endophytes] initiates a signal exchange which prepares the partners for the interaction. Root-derived flavonoids activate the release of factors from the microbes, which induce calcium spiking in root hairs [1]. Downstream of calcium spiking, reprogramming of gene expression in the roots induces mycorrhiza or nodule formation or the establishment of a beneficial mutualistic interaction [2,3]. The symbiotic signals of mycorrhizal fungi, the Myc factors, and those from rhizobial bacteria, Nod factors, are lipo-chitooligosaccharides. They are perceived by lysin-motif (LysM) receptors which induce a signaling pathway leading to either mycorrhiza or nodule formation. Myc factors from *Glomus intraradices* reprogram root gene expression and induce root branching and mycorrhization in *Medicago truncatula* ([4]; and ref. therein). Interestingly, LysM receptors are also involved in the perception of chitooligosaccharides, fungal cell wall compounds that induce defense responses and resistance to pathogens. This raises the question of how

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plants (legumes) discriminate between beneficial and pathogenic microorganisms (cf. [5]). Furthermore, for the establishment of a mutualistic interaction, the beneficial fungi have to overcome the defense machinery of the host to develop within the host. Kloppholz et al. [6] showed that the AM fungus *G. intraradices* uses the effector protein SP7 to short-circuit the plant defense program. SP7 is secreted and interacts with the pathogenesis-related transcription factor ERF19 in the plant nucleus. *ERF19* is highly induced in roots by the fungal pathogen *Colletotrichum trifolii* as well as by several fungal extracts, but only transiently during mycorrhiza colonization. When constitutively expressed in roots, SP7 leads to higher mycorrhization while reducing the levels of *C. trifolii*-mediated defense responses. Therefore, SP7 is an effector that contributes to develop the biotrophic status of AM fungi in roots by counteracting the plant immune program. These examples show that the symbionts cross-talk *via* chemical mediators which are released into the rhizosphere, and these compounds can be effective prior to the physical contact of the symbionts.

We study the beneficial interaction between the root-colonizing fungus *Piriformospora indica* and the model plant *Arabidopsis thaliana*. The endophyte colonizes the roots of many plant species, and - similar to AM fungi - promotes plant growth, biomass and seed production and confers resistance to abiotic and biotic stress ([7,8]; and references therein). *P. indica* is a member of Sebacinales, grows inter- and intracellularly and forms pear shaped spores, which accumulate within the roots and on the root surface. After the establishment of a beneficial interaction barely any defense or stress genes are activated and no reactive oxygen species (ROS) are produced by the host against *P. indica* [8,9]. Prior to the establishement of a symbiotic interaction and a physical contact between the two partners, *P. indica* releases exudate compounds, which induces appropriate responses in the host. For instance, a fungal compound induces cytoplasmic calcium ($[Ca^{2+}]_{cyt}$) elevation in the roots of Arabidopsis and *Nicotiana tabacum*, which is important for establishing the proper host response to the microbe. $[Ca^{2+}]_{cyt}$ elevation is followed by a nuclear Ca^{2+} response in the root cells [3]. Rafiqi et al. [10] presented a list of putative effector molecules which were identified in the *P. indica* genome and which might be secreted in order to modulate host cell's function and structure and to promote microbial growth on plant tissue. Finally, *P. indica* releases small molecular compounds into the medium and the root environment which prevent growth of pathogenic fungi and thereby restrict their growth also in the roots [11].

We have established standardized co-cultivation conditions of *P. indica* and Arabidopsis seedlings on Petri dishes which allow us to investigate the information exchange and the establishment of the mutualistic interaction between the two partners [12]. Here, we report that the seedlings respond to the presence of the fungus as early as two days after co-cultivation although the two organisms have not yet established a physical contact. After six days the hyphae and roots have contact to each other and the first hyphae are detectable within the exodermis of the roots. We report that both roots and leaves respond to the presence of *P. indica* already two days after co-cultivation. The response pattern is quite different four days later, when the hyphae have contact to the roots.

Results

Co-cultivation conditions of *P. indica* and Arabidopsis

An agar plaque with *P. indica* mycelium and an Arabidopsis seedling were transferred to a nylon membrane on solidified PNM medium on a Petri dish, with a distance of 3 cm. As control, an agar plaque without fungal hyphae was used (Additional file 1: Figure S1A). Under these co-cultivation conditions, the fungal mycelium and the roots start to grow but they have no contact to each other within the first two days of co-cultivation (Additional file 1: Figures S1B; S2A, B). At this time point, both organisms are separated by at least two cm. Therefore, any communication between the two organisms is only possible *via* exudated soluble compounds into the medium or through the gas phase. After six days of co-cultivation the growing roots and hyphae have reached each other and a physical contact has been established (Additional file 1: Figures S2C, D1, D2). Light and fluorescent microscopical analyses demonstrate that the mycelium penetrates the epidemal layers of the root. Formation of the first fungal spores around the roots becomes also visible (Additional file 1: Figures S2D1, D2). We measured defense and symbiotic responses of the seedlings during the first 14 days of co-cultivation (0, 1, 2, 4, 6, 10, 14 days). After 2 days of co-cultivation, a strong difference in the responses of *P. indica*-exposed and mock-treated control was detectable. After 6 days of co-cultivation, the response pattern was different from that observed at the earlier time point, and did not change much after longer co-cultivation (14 days). We reasoned that the early changes are induced by chemical mediators from the fungus, and that the later changes occur once a physical contact between the two symbionts is established. Therefore, we analysed the response of the roots to the presence of *P. indica* after two and six days of co-cultivation in more details.

Stomata aperture

Although a physical contact between the two partners has not yet been established after two days, the leaves of

the seedlings respond to the presence of the fungus by closing the stomata (Figure 1). Prior to expose to *P. indica*, 14.6 ± 1.1% of the stomata in the leaves were closed. Almost identical results were obtained for seedlings exposed to an agar plaque without the fungus for either two or six days (two days: 13.9 ± 3.3%; six days: 12.9 ± 3.7%). In contrast, two days after exposure of the seedlings to the *P. indica*-containing plaque, 76.7 ± 2.9% of the stomata were closed. Longer co-cultivation resulted in re-opening of the stomata, and after six days, only 17.5 ± 1.2% of the stomata remained closed (Figure 1). This demonstrates that regulation of stomata opening in the leaves in response to the root-colonizing fungus *P. indica* is a sensitive marker for the interaction of the two partners. To clarify whether the fungal signal (s) is an exudated compound in the medium or a gas, we co-cultivated Arabidopsis seedlings with *P. indica* on split Petri dishes. Exudated compounds from the fungus in the medium cannot reach the roots, while communication *via* gases or volatiles is possible. The number of

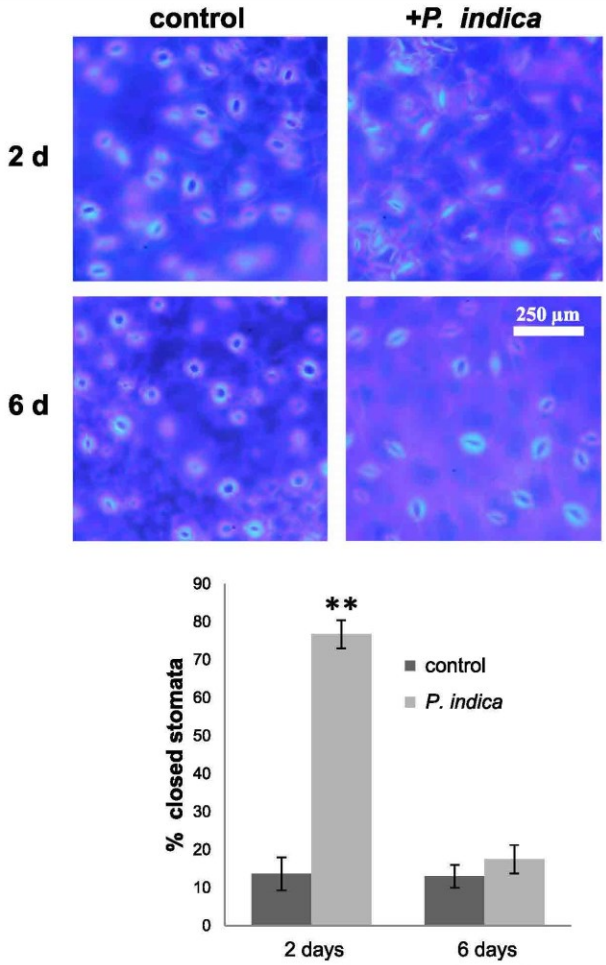

Figure 1 Epidermis with stomata of Arabidopsis leaves two or six days after exposure of the seedlings to an agar plaque without (control) or with *P. indica* (+ *P. indica*). Guard cells were visualized under the fluorescent microscope (450-520 nm) after stained with calcoflour white (the upper level). The lower panel shows the % closed stomata. Based on 3 independent biological experiments with 10 leaves from individual seedlings each. Bars represent SEs. Asterisks indicate significant differences, as determined by Student's *t*-test (**P < 0.01).

closed stomata in Arabidopsis seedling was not significantly different two days after co-cultivation of the symbionts on the split Petri dishes compared to the mock-treated control (control: 18.00 ± 1.65%; split Petri dishes: 18.87 ± 2.17%) which excludes gases and volatiles as chemical mediators.

H_2O_2 production

High doses of the fungus did not stimulate H_2O_2 production in roots and shoots [9] which has been confirmed for roots exposed to *P. indica* for six days (Figure 2). In contrast, two days after co-cultivation, we observed a higher H_2O_2 level in the leaves of *P. indica*-exposed seedlings compared to the mock-treated controls (Figure 2). This suggests that exudated compounds from the fungus trigger ROS production, and this stimulatory effect is no longer detectable six days after co-cultivation. Separation of the mycelium from the roots in split Petri dishes prevented the stimulation of H_2O_2 production after two days of co-cultivation (control: 0.0033 ± 0.0014 μg/mg dry weight; + *P. indica*: 0.0027 ± 0.0013 μg/mg dry weight), which again supports the involvement of a diffusible compound in the medium.

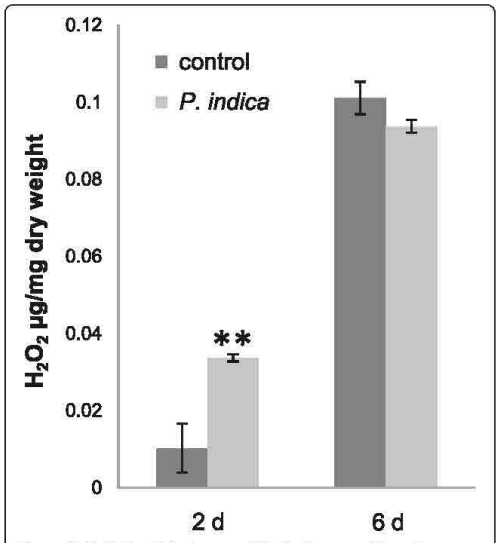

Figure 2 H_2O_2 levels in leaves of *A. thaliana* seedlings two or six days after exposure to an agar plaque without or with *P. indica*. The amount of μg H_2O_2 per mg dry tissue was determined as described in METHODS. Based on 3 independent biological experiments with 10 leaves from individual seedlings. Bars represent SEs. Asterisks indicate significant differences, as determined by Student's *t*-test (**P < 0.01).

Regulation of *NTR2.5* in the leaves in response to *P. indica*

NRT2.5 belongs to the nitrate transporter family and is preferentially, but not exclusively, expressed in leaves. The protein plays an essential role in plant growth promotion by the rhizospheric bacterium strain *Phyllobacterium brassicacearum* STM196 [13,14]. The regulation of its mRNA level in the leaves appears to be very sensitive to signals from the roots. Figure 3 demonstrates that the mRNA level for NRT2.5 in the roots is ~ 4-6-fold up-regulated by *P. indica*, two and six days after co-cultivation. Furthermore, while no significant response can be detected in the leaves two days after co-cultivation, a ~4-fold up-regulation is observed six days after co-cultivation of the seedlings with *P. indica*. This shows that signals from the fungus are transferred to the leaves, although the response is slower than this for stomata closure (Figure 1) and ROS production (Figure 2). The *NRT2.5* mRNA levels in the roots and leaves on split Petri dish experiments were not up-regulated in comparison to the mock-treated controls (data not shown) which again demonstrates that the *NTR2.5* response is mediated by fungus-derived non-gaseous chemical mediators.

Phytohormone levels in Arabidopsis roots and shoots two and six days after co-cultivation with *P. indica*

Beneficial plant-microbe interactions are associated with changes in phytohormone levels [15-17]. In order to test

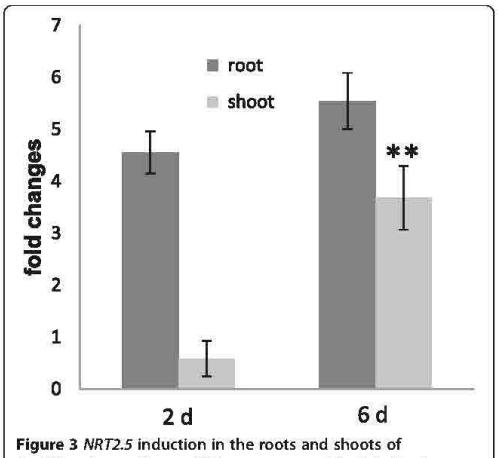

Figure 3 *NRT2.5* induction in the roots and shoots of Arabidopsis seedlings which were exposed to *P. indica* for either two or six days. The fold change relative to the mock-treatment is presented. Based on 3 independent biological experiments with 3 technical replicates each. Bars are SEs; they represent the sum of the SEs of the individual values. Asterisks indicate significant differences (six day shoot value *vs.* two day shoot value), as determined by Student's *t*-test (**P < 0.01).

whether co-cultivation of Arabidopsis roots with *P. indica* affects the phytohormone levels, the amounts of jasmonic acid (JA) and its active form JA-isoleucine (JA-Ile), 12-oxo-phytodienoic acid (OPDA), abscisic acid (ABA) and salicylic acid (SA) were determined in the roots and shoots of seedlings either exposed to *P. indica* or mock-treated. Interestingly, we observed the strongest up-regulation of the phytohormone levels in both roots and shoots two days after co-cultivation. The phytohormone levels decreased significantly in both roots and shoots after six days of co-cultivation (Figure 4). Since the hormones are involved in various types of stress and defense responses, the results indicate that exudated compounds from the fungus induce stress hormones in the roots and systemically also in the leaves. Their level declines as soon as a physical contact between the two organisms is established.

Transcriptome analyses for Arabidopsis roots two and six days after exposure to *P. indica*

Roots exposed to *P. indica* for two and six days were harvested for RNA extraction and expression profiling. Root material exposed to agar plaques served as control. Only genes from *P. indica*-exposed material which showed a > 3-fold difference to the agar control were analysed in this study. The comparative transcriptome analysis [18] uncovered that 75 genes were up-regulated and 14 genes down-regulated after two days, whereas 50 genes were up-regulated and 4 genes down-regulated after six days (Figure 5; Figure 6; Additional file 1: Table S1A, C). Categorization of the genes using the Mapman software revealed a huge difference between the two datasets.

Thirthy-five stress- and defense-related genes are only up-regulated during the early time point of co-cultivation and thus appear to respond to chemical mediators released by the fungus (cf. Discussion; Figure 5; Figure 6; Additional file 1: Table S1A). This includes genes for defense-related cell wall proteins and transcription factors, subtilase At1g32940 [19], a protease inhibitor, chitinase, germin-like protein, PAD3, CYP71B6, galactinol synthase 4, glycosyltransferase 73D1, leucine-rich repeat proteins, glutathione-S-transferases (GST) and glutaredoxin 480. Furthermore, phytohormone-

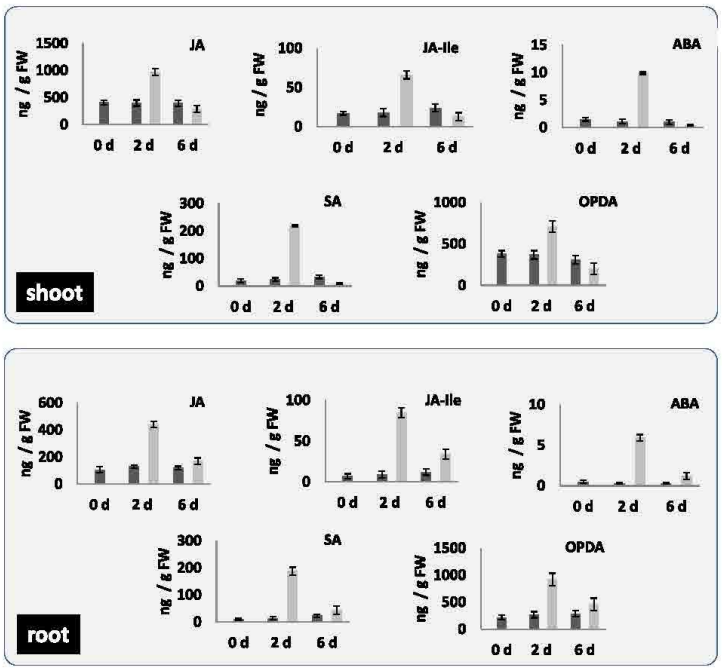

Figure 4 Phytohormone levels in roots and leaves of Arabidopsis seedlings after exposure to *P. indica* for two or six days. The roots and shoots of the seedlings were harvested at day 0, 2 and 6 after exposure to the *P. indica* plug or an agar plug without mycelium. SA, ABA, JA, *cis*-OPDA and JA-Ile levels were determined. The values are means ± SEs of 4 independent biological experiments with 5 replications in each experiment.

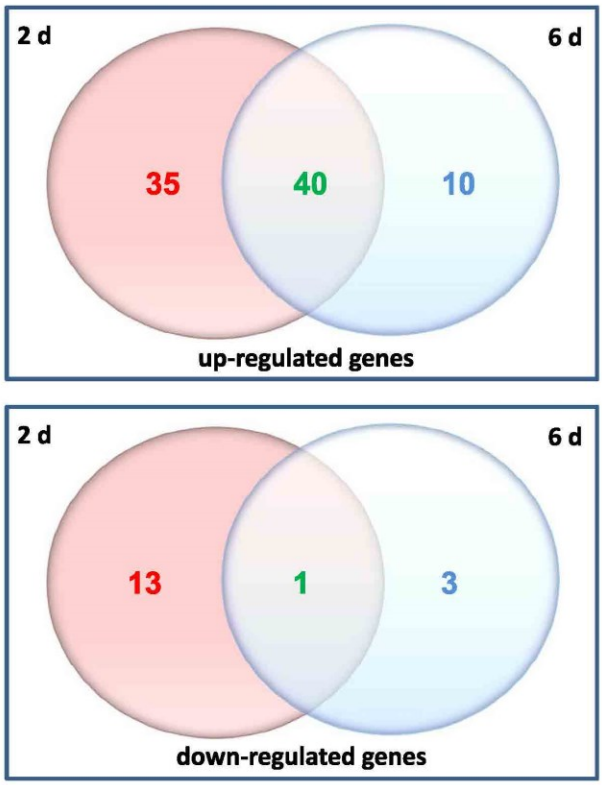

Figure 5 Venn datagram of the number of genes which are up- or down-regulated in Arabidopsis roots exposed to *P. indica* for either two or six days. Numbers of genes regulated only after 2 d of interaction are shown in red colour; those regulated only after 6 d are shown in blue; number of genes regulated at both time points are shown in green. The results are based on 3 independent biological experiments.

related genes such as *CYP81D8* (At4g37370), *CYSTEINE PROTEINASE1* (At4g36880), *GH3.4* (At1g59500), *TOUCH3* (At2g41100) and those with Ca^{2+}-related functions [*CIPK13* (At2g34180) and At4g33050] are also up-regulated two days after co-cultivation (cf. Discussion). In contrast, genes involved in developmental and DNA modifications, such as *HISTONE H1-3*, *PYRIDOXINE BIOSYNTHESIS1.1* and *CML38* are down-regulated.

The number of defense- and stress-related genes is much less after six days of co-cultivation (cf. Discussion).

The majority of the identified genes are regulated by *P. indica* at both time points (Figure 5; Additional file 1: Table S1B). Closer inspection of the expression levels of these genes also confirms a decline in the degree of defense processes from the 2^{nd} to the 6^{th} day after co-cultivation (cf. Discussion). Examples are genes for the root-specific proline-rich extensin At1g26240, PHOSPHOLIPASE A 2A (At2g26560), GERMIN-LIKE PROTEIN19, CYP81F2, chitinase At2g43570, the disease resistance protein At2g15120, ENDOPEPTIDASE INHIBITOR1 (At2g43510), the Ca^{2+}-binding proteins At5g26920 and At5g39670, the transferase At5g42830, the NAC domain transcription factor JUNGBRUNNEN1, ERD11, ACIREDUCTONE DIOXYGENASE3 and GLUTHATIONE S-TRANSFERASE TAU10 (cf. Discussion). The lower expression level during later stages of co-cultivation indicates that the gene products are less required once a physical contact has been established between the two symbionts.

For 33 randomly chosen genes from the three categories (Additional file 1: Table S1A-C), the microarray results were confirmed by qRT-PCR analyses. Additional file 1: Table S1D demonstrates that most of the results confirmed the microarray data.

	2 days		2 and 6 days		6 days	
	↑	↓	↑	↓	↑	↓
DNA synthesis		1				
Protease inhibitor		1				
Co-factor and vitamin metabolism		1				
Light signaling		2		1		
UDP glucosyl and glucoronyl transferases	1	2				
Calcium signaling	3	1	2			
Development, unspecified	1	1	2			
Hormone metabolism, auxin	1	1	1		1	
Secondary metabolism	1	1	2		1	
Protein degradation, subtilases	2		1		1	
Biotic stress	3		5		1	
Nitrilases, reticuline oxidases, troponine reductases	1		2			
Glutathione S transferases	2		4			
Cytochrome P450	3		1			1
Amino acid metabolism, tryptophan	1					
Plastocyanin-like	1					
RNA, regulation of transcription	4					
Minor CHO metabolism	1					
Protein synthesis, ribosomal protein, eukaryotic, 60S subunit L34	1					
Protein, post-translational modification	1					
Abiotic stress, unspecified	2					
Redox, glutaredoxins	1				1	
Signaling, receptor kinases	1				1	
Peroxidases			2			
GDSL-motif lipase			1			
Cell wall proteins, HRGP			1			
Lipid metabolism, lipid degradation			1			
Signaling, MAP kinases			1			
Cell organization			2			
O-methyl transferases			3		1	
Metal transport					1	1
Fermentation, ADH					1	
Glycolysis						1
Not assigned, unknown	5	3	10		1	

Figure 6 Number of genes of the MAPMAN categories which are either up-regulated (blue) or down-regulated (red) in Arabidopsis roots 2 or 6 or [2,6] days after co-cultivation with *P. indica*. 2 days: genes which are regulated only after 2 days of interaction; 6 days: genes which are regulated only after 6 days of interaction; [2 and 6 days]: common genes which are regulated at both time points. The results are based on 3 independent biological experiments. For detailed information, cf. Additional file 1: Table S1.

To clarify the nature of the fungal signal(s) which modifies the root transcriptome pattern under short term co-cultivation (2 days), we performed co-cultivation experiments on split Petri dishes as described above. The transcriptome pattern of the randomly chosen 33 genes was studied using real-time PCR (Additional file 1: Table S2), but no significant difference was observed to the mock-treated control (Additional file 1: Table S2). This demonstrates again that gases and volatiles do not play a role in changing the gene expression patterns in Arabidopsis roots. Apparently, diffusible compounds released by the hyphae are required for the observed reprogramming of the root transcriptome.

Discussion

Diffusible compounds released by microbes trigger plant responses before physical cell-to-cell contact occurs [1,20-22]. Several lines of evidence demonstrate that *P. indica* releases compounds which induce defense processes in Arabidopsis roots. The identified genes which are up-regulated after two days of co-cultivation and their role in plant/microbe interaction support this idea. Since the mycelium has not yet reached the roots, plant responses must be induced by either chemical mediators secreted into the medium or gaseous compounds. The split Petri dish experiments support the first possibility, although it cannot be excluded that gaseous compounds also participate in the communication. We also failed to

identify major volatile organic compounds which are released into the air in the *P. indica*/Arabidopsis root symbiosis (D. Tholl and R. Oelmüller, unpublished).

Exudate compounds from both fungal mycelium and roots are well characterized mediators of early communication in mycorrhizal symbiosis [23-25]. The exudate from AM fungi induces also nitric oxide (NO) accumulation in *Medicago truncatula* roots [26]. NO is involved in control of stomata closure ([27]; and ref. therein), therefore, fungus-induced and plant-released NO could be involved in the regulation of stomata aperture. The early plant responses in the leaves (stomata closure and ROS production) could be caused by NO of plant origin, which is synthesized in response to chemical mediators released from *P. indica* before a physical contact has been established.

Stomata closure is a typical ABA-mediated stress response, which might be induced by exudated signals from *P. indica*. Many bacterial pathogens invade plants primarily through stomata on the leaf surface. Sawinski et al. [28] showed that microbial invasion is restricted or prevented by stomata closure upon perception of MAMPs, and this represents an important layer of active immunity at the preinvasive level. The signaling pathways leading to stomatal closure triggered by biotic and abiotic stresses employ several common components, such as ROS, Ca^{2+}, kinases and hormones, suggesting considerable interaction between MAMP- and ABA-induced stomatal closures. Entry of the foliar pathogen *Pseudomonas syringae* pathovar tomato DC3000 into the plant corpus occurs also through stomatal openings, and consequently a key plant innate immune response is the transient closure of stomata. Kumar et al. [29] showed that root colonization by the rhizobacteria *Bacillus subtilis* FB17 restricts the stomata-mediated pathogen entry of PstDC3000 in Arabidopsis and root binding of FB17 invokes ABA and SA signaling to close the stomata. These results emphasize the importance of rhizospheric processes and environmental conditions as an integral part of the plant innate immune system against foliar bacterial infections, and similar processes may occur in the system described here.

We have previously demonstrated that colonization of Arabidopsis roots by *P. indica* does not result in H_2O_2 production [3,8]. Like the regulation of stomata closure, ROS production is fast in response to fungal signals. ROS is also produced during early stages of symbiotic interactions of bacteria and mycorrhizal fungi with roots [30,31]. Here, we demonstrate an early production of ROS before a physical contact between the two symbionts has been established. This is likely initiated by exudated compounds from the fungus. They can function as PAMPs, similar to PAMPs released by pathogenic fungi which activate ROS production *via* activation of the root NADPH oxidase or apoplastic peroxidases, or by gaseous compounds. Our results with split Petri dishes argue against a role of gaseous compounds in this response (Additional file 1: Table S2). These ROS could activate the observed defense responses at the mRNA level, both locally and systemically, two days after co-cultivation of the two symbionts. Fungi also contain NADPH oxidases [32]. *Epichloe festuca*-synthesized ROS regulate hyphal tip growth, thereby restricting growth of the fungus and preventing excessive colonization and host defense gene activation [31,32]. Accumulation of ROS, the oxidative damage to lipids and the membrane electrolyte leakage is lower in AM plants than in non-mycorrhizal plants [33,34], presumably due to the efficient up-regulation of ROS scavenging systems.

Six, but not two days after co-cultivation, we observed the up-regulation of the *NRT2.5* mRNA level in the leaves, indicating a slow root-to shoot signal transduction process in the presence of the fungus. Like *P. indica*, Arabidopsis growth is stimulated by the *Phyllobacterium brassicacearum* STM196 strain, and this is associated with the up-regulation of *NRT2.5* and *NRT2.6* [14]. The *nrt2.5* and *nrt2.6* mutations abolished plant growth and root responses to STM196. Thus, *NRT2.5* and *NRT2.6*, which are preferentially expressed in leaves, play an essential role in plant growth promotion by the rhizospheric bacterium STM196. Members of the *NRT2* family have also been described to be involved in plant defense responses: *NRT2.1* in the priming against *Pseudomonas syringae* pv *tomato* [35] and *NRT2.6* in the resistance against *Erwinia amylovora* [36]. Both genes are required for STM196-induced plant growth promotion, and thus represent new genes in beneficial biotic interactions. Furthermore, these genes participate in a pathway that alters the classically described regulation of shoot - root biomass allocation and root development through the plant nitrogen status. The exact role of these genes in the *P. indica*/Arabidopsis symbiosis remains to be determined, however, *NRT2.5* is a sensitive leaf marker for *P. indica* colonization of the roots.

Phytohormones play important roles in almost all types of plant-microbe interactions. We demonstrate that the defense-related phytohormones JA, Ja-Ile, ABA, SA and OPDA are strongly up-regulated during early phases of co-cultivation of *P. indica* with Arabidopsis roots. Since no physical contact has been established at this time point, their up-regulation must be induced by exudated signals from the fungus (Figure 4). Mukherjee and Ané [37] reported that ethylene inhibits induced symbiotic gene expression and root development in response to germinating spore exudates in mono- and dicots. We observed a quite strong up-regulation of ABA in both roots and leaves in response to secreted fungal compounds (Figure 4). It is consistent with the

observed closure of the stomata at this time point. Herrera-Medina et al. [38] reported lower colonization of the roots of the ABA-deficient mutant *sitiens* in tomato. Furthermore, the arbuscules were also less developed in the mutant, and both lesions could be restored by exogenous application of ABA to the *sitiens* mutant. It appears that ABA is essential for full AM colonization and arbuscule development (cf. [38]). ABA may down-regulate arbuscular formation directly [39], e.g. by stimulating genes involved in defense and cell wall modification [21], or indirectly by stimulating ethylene production [39]. Garrido et al. [40] showed significant differences in gene expression in mycorrhizal roots of wild-type (WT) and ABA-deficient tomato mutants, and these differences corresponded to the ABA content in the roots. Our data support the important role of ABA in beneficial plant/microbe interactions. Up-regulation of components involved in ABA processes has also been reported by Schäfer et al. [41] in the *P. indica*/barley interaction.

JA, JA-Ile and OPDA are well characterized hormones involved in pathogen attack [42]. Their participation in beneficial plant-microbe interactions is quite controversial (cf. [43]). We observed a strong up-regulation of all these hormonal compounds during early phases of the co-cultivation which is consistent with the observation that JA-regulated stress genes are also up-regulated during the early co-cultivation period. Regvar et al. [44], Isayenkov et al. [45] and Landgraf et al. [46] showed a promotion and Ludwig-Müller et al. [47] a reduction of AM colonization in response to JA or JA-Ile in different systems. Tejeda-Sartorius et al. [48] showed that AM colonization was reduced in a JA-deficient tomato mutant [49], and the lesion could be restored by methyl JA application. In contrast, Herrera-Medina et al. [50] showed that the JA-insensitive *jai-1* tomato mutant showed increased colonization and the WT tomato was less colonized after methyl JA application. *Nicotiana attenuata* plants silenced for *COI1* expression showed elevated AM colonization [51]. In spite of quite different results, it appears that JA plays a crucial role in beneficial plant-microbe interactions. JA exogenously applied to the growth medium also decreases the number of nodules induced by *Sinorhizobium meliloti* on *Medicago truncatula* roots [52]. JA decreases the responsiveness of Ca^{2+} spiking to Nod factor application and high concentrations of JA inhibited spiking [52], and this might affect root colonization. Application of JA and methyl JA to roots induced the expression of *Nod* genes [53] and the production of Nod factors [54]. This suggests that JA is not exclusively involved in the activation of defense responses. The lower level of JA, JA-Ile and OPDA six days after co-cultivation indicates that these compounds play a less dominant role once the partners have recognized themselves as friends. This resembles reports by Kouchi et al. [55] who showed that during early phases of colonization of *Lotus japonicus* roots by *Mesorhizobium loti* JA-biosynthesis genes are up-regulated. After initiation of nodule formation, they were repressed again.

SA is mainly required for the plant's defense against biotrophic pathogens (cf. [56]). We observed a strong response in both roots and shoots, but it is not different from the JA, JA-Ile and OPDA responses (Figure 4). An increase in the SA level has also been reported during early stages of AM colonization [57], and this might be important for root colonization by AM fungi [58]. The transient increase in the SA level induces SA-responsive defense genes in *Medicago truncatula* roots at early stages of AM colonization [59], similar to the result described here. Tobacco plants with higher SA levels showed reduced root colonization at early time points, but this effect disappeared during later phases of the interaction [50]. How the defense responses induced by the elevated phytohormone levels are down-regulated when a physical contact between the two symbionts has been established remains to be determined. JA signaling might counteract SA signaling at early stages of the recognition of the two symbionts.

Many genes involved in plant defense are regulated during the co-cultivation of Arabidopsis roots with *P. indica*, however there are clear differences between the early and later time points. Many defense related genes are regulated two and six days after co-cultivation, although their stimulation is lower at the later time point. 35 genes which were up-regulated after 2 days co-cultivation with *P. indica* are stress and defense genes. The germin-like protein 4 (At1g18970) exhibits superoxide dismutase activity and its homologs in barley and wheat are important resistance component against *Blumeria graminis* [59]. The defense-related *WRKY54* [60], *WRKY70* (At3g56400) and *MYB51* (At1g18570) transcription factor genes are involved in basal resistance, stress tolerance [60] or secondary metabolite synthesis [61]. The oxygenic stress-inducible aspartyl protease At3g59080 [62], the HOPZ-ACTIVATED RESISTANCE1 leucine-rich repeat protein (ZAR1, At3g50950) [63], the protease YLS5, the leucine-rich repeat protein kinase At1g51890, the VQ motif protein At4g20000, the WD40 protein (At5g42010, TAIR homepage) and PAD3 (At3g26830, CYP71B15) for camalexin biosynthesis (cf. [64]) participate in different aspects of plant immunity or are induced by pathogen treatments. Several glutathione-S-transferase (GST) genes are also up-regulated at the early time point of interaction. *GSTF3* (At2g02930) responds to *Fusarium sporotrichioides* infection [65] and *GSTL1* (At5g02780) to a wide range of chemicals and abiotic stress treatments [66]. *GST2*, a

Ca^{2+}-ATPase (At3g63380) is activated by fungal and nematode stimuli and stress (TAIR homepage). Phytohormone-related genes are also up-regulated by chemical mediators from *P. indica*. The antranilate synthase subunit α1 is important for JA-mediated regulation of auxin biosynthesis and transport during lateral root formation [67], GH3.4 (At1g59500) plays an important role in auxin homeostasis [68], the JA-regulated *CYP81D8* (At4g37370) product is involved in phenylpropanoid biosynthesis [69,70], *CYSTEINE PROTEINASE1* (At4g36880) responds to gibberellin [71], and *TOUCH3* (At2g41100) to SA [72,73]. We conclude that many genes which were up-regulated in response to the fungal exudates, code for defense and stress proteins, compounds involved in signaling leading to defense gene activation or control phytohormone homeostasis.

14 genes which are down-regulated two days after co-cultivation with *P. indica* are involved in developmental processes and DNA metabolism. *HISTONE H1-3* (At2g18050) encodes a linker histone protein whose expression is stimulated by dehydration and ABA [74]. *PYRIDOXINE BIOSYNTHESIS1.1* (At2g38230) controls plant growth, development and stress tolerance [75]. *At4g12550* is an auxin-induced gene in roots. CML38 (At1g76650) is involved in Ca^{2+} signaling and important for Ca^{2+}-mediated developmental and stress responses and epidermal development or morphology [76]. The plastid-localized CCL protein (At3g26740) is controlled by the circadian clock during the day [77].

Only ten stress- and defense-related genes are up-regulated six days after co-cultivation. Among them are *ALCOHOL DEHYDROGENASE1* (*ADH1*), which is up-regulated in roots by osmotic stress [78] and ABA [79], the ethylene-responsive transcription factor gene *ERF105* (At5g51190) which responds to chitin treatment [80], and *INDOLE GLUCOSINOLATE O-METHYLTRANSFERASE1* (At1g21100) involved in hydroxylation reactions of the glucosinolate indole ring [81]. The *L-ascorbat oxidase At4g39830* gene is inducible by pathogens [82] and MILDEW RESISTANCE LOCUS6 mediates defense response to fungi and cell death [83]. Genes related to developmental processes code for the AAA-ATPase (At5g40010) which participates in plastidial transport [84], for the CAFFEOYL-COA 3-O-METHYLTRANSFERASE (At1g67980) which catalyses lignin monomer biosynthesis [85], and the CATION/H^+ EXCHANGER17 (At4g23700) which regulates cation and pH homeostasis [86].

The group of common genes which are regulated at both time points includes the NAC domain transcription factor gene *JUNGBRUNNEN1* which is induced by H_2O_2 [87], GDSL LIPASE1 (At5g40990) that plays an important role in plant immunity [88], ERD11 (At1g02930) and the GLUTHATIONE S-TRANSFERASE TAU10 (At1g74590) which are induced by oxidative stress and bacterial infections (TAIR homepage). *ACIREDUCTONE DIOXYGENASE3* (At2g26400) which functions in H_2O_2 and SA signaling, is induced by hypoxia and involved in systemic acquired resistance (TAIR homepage). The oxidoreductase *At4g10500* gene is induced strongly when Arabidopsis seedlings are grown on a *P. indica* lawn [9]. Also At5g38900 (DSBA oxidoreductase) and At2g18690 have been described to be involved in defense against pathogenic fungi. All these genes are stronger up-regulated in Arabidopsis roots before a physical contact has been established between the two symbionts, which suggest that they are induced by *P. indica*-released chemical mediators.

Comparison of transcripts in rice roots, which were colonized by AM Glomalean fungi with those colonized by pathogens (*Magnaporthe grisea* and *Fusarium moniliforme*) showed that over 40% of the genes were differentially regulated by both the symbiotic and at least one of the pathogenic microbes. Güimil et al. [89] proposed that the common genes may play a role in compatibility. Furthermore, 34% of the mycorrhiza-associated rice genes were also associated with mycorrhiza in dicots, revealing a conserved pattern of response between the two angiosperm classes. Campos-Soriano and Segundo [90] hypothesized that increased demands for sugars by the fungus might be responsible for the activation of the host defense responses which will then contribute to the stabilization of root colonization by the AM fungus. However, the precise role of defense responses in mutualistic interactions is not clear. Excess root colonization might change a mutualistic association into a parasitic association (cf. [31]). This argues in favor of a role of plant defense compounds in restricting root colonization, thereby stabilizing the symbiotic interaction. Studies with the *P. indica*/Arabidopsis symbiosis support the idea [16,91]. However, inoculation with *G. intraradices* stimulated growth and biomass production in WT rice plants and plants overexpressing defense genes. The fungus activates basal defense response in mycorrhizal rice roots, including PR proteins and antioxidant enzymes. Although constitutive expression of defense genes occurred in the roots of the overexpressor lines, the symbiotic efficiency of *G. intraradices* in these plants was not affected. These results suggest that AM fungi have evolved the capacity to circumvent defense mechanisms that are controlled by the plant's immune system [92]. Similar observations have been described for the *P. indica*/Arabidopsis interaction [93]. The authors demonstrate that a broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by *P. indica*.

Conclusions

In conclusion, our data (Figure 7) suggest that *P. indica* releases chemical compounds prior to a physical contact which activate stress and defense processes in the host (2 days). Apparently, pre-contact signaling molecules prepare the plant for the symbiotic interaction, and activation of defense may be the first line of recognition. The plant response is not restricted to roots, but also detectable in the leaves. Once root colonization has taken place (6 days) defense responses are down-regulated and genes involved in promoting plant growth, metabolism and performance are activated.

Methods

Growth conditions of *A. thaliana* and fungi

A. thaliana WT (ecotype Columbia-0) seeds were surface-sterilized and placed on Petri dishes containing MS nutrient medium [94]. After cold treatment at 4°C for 48 h, plates were incubated for 10 days at 22°C under continuous illumination (65 μmol m^{-2} sec^{-1}). *P. indica* was cultured for three weeks at 22-24°C on *Aspergillus*-minimal medium [95,96 (Section A)].

Co-cultivation of seedlings with *P. indica*

Twelve day-old (48 h cold treatment and 10 days of illumination) Arabidopsis seedlings of equal sizes were selected for co-cultivation experiments. They were transferred to PNM plates with a nylon membrane on the top [96 (Section C-Method 1)] and exposed to a fungal plug 5 mm in diameter or a KM plug of the same size without fungal hyphae (control). The plugs were placed 3 cm away from the closest root part (Additional file 1: Figure S1A). The experimental setup was identical on the split Petri dishes, except that the fungal (or control) plaques were placed on one side and the seedlings on the other side of the Petri dish. The light intensity (80 ± 5 μmol m^{-2} sec^{-1}) was checked every third day to ensure that both *P. indica*- and mock-treated seedlings receive equal amounts of light.

Gene expression

Total RNA was isolated separately from roots and shoots of WT seedlings after two and six days of co-cultivation (or mock-treatment) with *P. indica* using RNeasy Plant Mini Kit (Qiagen). After reverse-transcription, cDNA was synthesized from 1 μg total RNA using the Omniscript RT Kit (Qiagen) and oligo (dT)20 in 20 μl reaction volume. Real-time quantitative PCR was performed with gene-specific primers (Additional file 1: Table S3) and performed using the CFX connect Real-time system and the CFX manager software version 3.1 (Bio-Rad). For the amplification of the PCR products, iQ SYBR Supermix (Bio-Rad) was used according to the manufacturer's instructions in a final volume of 20 μl. The iCycler was programmed to 95°C 2 min, 35 × (95°C 30 s, 55°C 40 s, 72°C 45 s), 72°C 10 min followed by a melting curve (55-95°C in increasing steps of 0.5°C). All reactions were repeated twice. The mRNA

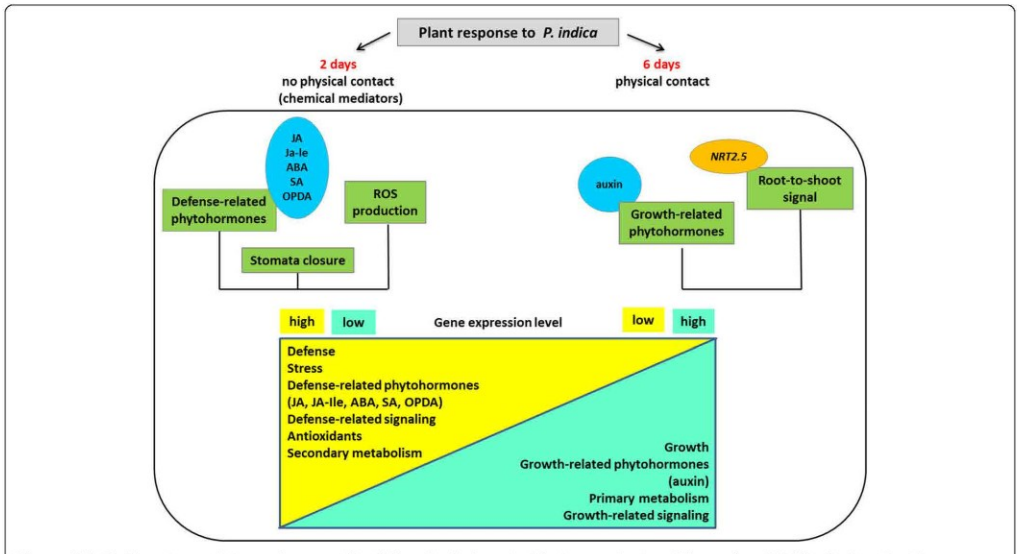

Figure 7 *A. thaliana* transcriptome changes after 2 (no physical contact between plant and fungus) and 6 (physical contact is established) days of co-cultivation with *P. indica*.

levels for each cDNA probe were normalized with respect to the *GAPC2* message levels. Fold induction values were calculated with the ΔΔCP equation of Pfaffl [97]. The ratio of a target gene was calculated in the *P. indica*-treated sample *versus* the mock-treated control in comparison to the *GAPC2* reference gene.

Microarray analyses, data processing

Microarray hybridizations for *P. indica*-exposed and mock-treated Arabidopsis roots were performed with the Arabidopsis Genome Array ATH1 (Affymetrix, USA) at the Kompetenzzentrum für Fluoreszente Bioanalytik, Regensburg, Germany. The hybridization signal data were analyzed with ROBIN (http://mapman.gabipd.org/web/guest/robin-download) and MapMan (http://mapman.gabipd.org/web/guest/robin-download) programs. Statistical analysis for *t*-test and subsequent calculation of false discovery rate were performed according to ROBIN program. The microarray data given in the Supplementary Material are based on 3 biological independent experiments. The results have been submitted to GEO (http://www.ncbi.nlm.nih.gov/geo, submission number GSE58771). The *NRT2.5* data shown here are based on Real-time PCR, since the gene was not present on all microarray chips.

Visualization of the cellular pathways and functional categories of the expression data of Arabidopsis roots after two and six days of co-cultivation with *P. indica* was carried out using the MapMan and Pegman package according to Ath_AFFY_ATH1_TAIR8_Jan 2010 (http://mapman.gabipd.org) [98]. The visualization Mapman tool was used to identify similarities and differences of different pathways involved in biotic and abiotic stress responses [98]. Wilcoxon test was used to visualize significantly expressed genes in Pegman. Venn diagrams were calculated using the expression log values of Mapman package [99]. Specifically expressed genes were determined by Venn diagram with a 3-fold change threshold. Also differentially regulated gene patterns were considered by Venn diagram according to comparative analysis of microarrays in the GEO microarray and NASC data sets.

Microscopy of roots and stomata staining

The roots of Arabidopsis seedlings exposed to *P. indica* for two or six days were stained with trypan blue and the colonization was analysed by light and fluorescent microscopy as described in Vahabi et al. [100]. Hyphae and spores in the roots could only be detected six days after co-cultivation of the two partners (Additional file 1: Figure S1B, S2C, D1, D2). For stomata staining, detached Arabidopsis leaves were stained using 1 ml calcoflour staining solution (10 mM calcoflour in 50% glycerol, 100 μm Tween 20) for 5 min, and the epidermal layers were analysed under a light and fluorescent microscope (450–520 nm). Opened and closed stomata from 5 areas in 10 leaves from different seedlings were counted. The data are averages of three independent biological experiments. Stomata are considered as closed when no open space can be seen between the two guard cells (Figure 1).

H_2O_2 and ROS measurements

Arabidopsis seedlings co-cultivated with *P. indica* for two and six days were stained with 3,3′-diaminobenzidine (DAB) as described by Daudi et al. [101]. As a result of staining a brown precipitate upon oxidation was formed, which is insoluble in aqueous and organic solvents [102,103]. For the detection/quantification of H_2O_2 inside the plant material, 100 mg of stained tissue was washed with acetone three times, ground to a fine powder and - after drying - dissolved in 1 ml DMSO at 90°C for 1 h. The supernatant was separated from the precipitate by centrifugation at 10,000 rpm for 5 min and was further used for spectrophotometric measurements at 270 nm (Perkin Elmer, Lambda 12) as described by Greenfield et al. [104]. The poly-DAB concentration of the plant tissue was correlated to the H_2O_2 concentration using a standard curve which was generated by the application of four different concentrations of H_2O_2 (0.1, 1, 10, 100 μg).

Phytohormone measurement

100 mg of leaf material was frozen in liquid nitrogen and kept at -80°C. After grinding with mortar and pestle, the leaf material was extracted with 1,2 ml of methanol containing 24 ng of 9,10-D_2-9,10-dihydrojasmonic acid, 24 ng D_4-salicylic acid (Sigma-Aldrich, Germany), 24 ng D_6-abscisic acid (Santa Cruz Biotechnology, Santa Cruz, USA), and 4,8 ng of JA-$^{13}C_6$-Ile conjugate as internal standards. JA-$^{13}C_6$-Ile conjugate was synthesized as described by Kramell et al. [105] using $^{13}C_6$-Ile (Sigma-Aldrich, Germany). The homogenate was mixed for 30 min and centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was collected. The homogenate was re-extracted with 500 μl methanol, mixed well, centrifuged and supernatants were pooled. The combined extracts were evaporated in a speed-vac at 30°C and re-dissolved in 250 μl methanol. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm; 1.8 μm; Agilent). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0-0.5 min, 5% B; 0.5-9.5 min, 5-42% B; 9.5-9.51 min 42-100% B; 9.51-12 min 100% B and 12.1-15 min 5% B. The mobile phase flow rate was 1.1 ml/min. The column temperature was maintained at 25°C. An API 3200

tandem mass spectrometer (Applied Biosystems) equipped with a Turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. The ionspray voltage was maintained at −4500 eV. The turbo gas temperature was set at 700°C. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 7 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: m/z 136.9 → 93.0 [collision energy (CE) - 22 V; declustering potential (DP) - 35 V] for SA; m/z 140.9 → 97.0 (CE - 22 V; DP - 35 V) for D4-SA; m/z 209.1 → 59.0 (CE - 24 V; DP - 35 V) for JA; m/z 213.1 → 56.0 (CE - 24 V; DP - 35 V) for 9,10-D2-9,10-dihydrojasmonic acid; m/z 263.0 → 153.2 (CE - 22 V; DP - 35 V) for ABA; m/z 269.0 → 159.2 (CE - 22 V; DP - 35 V) for D6-ABA; m/z 322.2 → 130.1 (CE - 30 V; DP - 50 V) for JA-Ile conjugate; m/z 328.2 → 136.1 (CE - 30 V; DP - 50 V) for JA-$^{13}C_6$-Ile conjugate. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies were verified by analyzing dilution series of standard mixtures. Phytohormones were quantified relative to the signal of their corresponding internal standard. For quantification of 12-oxophytodienoic acid, *cis*-OPDA, 9,10-D_2-9,10-dihydro-JA was used as the internal standard applying an experimentally determined response factor of 1.

Availability of supporting data

All the supporting data are included as Additional file 1.

Additional file

Additional file 1: Figure S1. Experimental design for the co-cultivation assays. **Figure S2**. Localization of *P. indica* mycelium and spores in and around Arabidopsis roots. **Table S1**. MAPMAN analysis of the genes which are regulated at least 3-fold in the roots of seedlings exposed to *P. indica* for two or six days. **Table S2**. Regulated genes in *A. thaliana* roots after two days interaction with *P. indica* grown on normal or split Petri dishes. **Table S3**. List of primers for RT-PCR used in this study.

Abbreviations

Pi: *Piriformospora indica*; ROS: Reactive oxygen species; MAMP: Microbe-associated molecular pattern; AM: Arbuscular mycorrhiza; JA: Jasmonic acid; SA: Salicylic acid; ABA: Abscisic acid; OPDA: 12-oxo-phytodienoic acid; JA-Ile: Jasmonic acid-isoleucine; WT: Wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KV designed and carried out most of the experiments. IS, MB, AM and AL helped in transcriptome analysis. MR did the phytohormone analysis. KV, IS and RO wrote the article. RO supervised the research. All authors read and approved the final manuscript.

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4.3 Manuscript III

Vahabi K, Karimi Dorcheh S, Monajembashi S, Sherameti I, Westermann M, Reichelt M, Falkenberg D, Hemmerich P, Oelmüller R (2015) Role of stress in Arabidopsis - *P. indica* interaction. Submitted to Mycorrhiza

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Mycorrhiza

Role of stress in Arabidopsis - P. indica interaction

--Manuscript Draft--

Manuscript Number:	MCOR-D-15-00044
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Abstract:	Abstract The endophytic fungus Piriformospora indica colonizes Arabidopsis roots and promotes plant performance, growth and resistance/tolerance against abiotic and biotic stress. Here we demonstrate that the benefits for the plant increase when the two partners are co-cultivated under mild stress (limited access to nutrient, exposure to heavy metals and salt, light and osmotic stress, pathogen infection). Moreover, physical contact between P. indica and Arabidopsis roots is necessary for optimal growth promotion, and chemical communication cannot replace the physical contact. Lower nutrient availability down-regulates and higher nutrient availability up-regulates the plant defense system including the expression of pathogenesis-related genes in roots. High light, osmotic and salt stresses support the beneficial interaction between the plant and the fungus. P. indica reduces stomata closure and H2O2 production after A. brassicae infection in leaves and suppresses the defense-related accumulation of the phytohormone jasmonic acid. Thus, shifting the growth conditions towards a mild stress promotes the mutualistic interaction, while optimal supply with nutrients or low stress diminishes the benefits for the plant in the symbiosis.

Manuscript III

Role of stress in Arabidopsis - *P. indica* interaction

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Abstract

The endophytic fungus *Piriformospora indica* colonizes Arabidopsis roots and promotes plant performance, growth and resistance/tolerance against abiotic and biotic stress. Here we demonstrate that the benefits for the plant increase when the two partners are co-cultivated under mild stress (limited access to nutrient, exposure to heavy metals and salt, light and osmotic stress, pathogen infection). Moreover, physical contact between *P. indica* and Arabidopsis roots is necessary for optimal growth promotion, and chemical communication

cannot replace the physical contact. Lower nutrient availability down-regulates and higher nutrient availability up-regulates the plant defense system including the expression of pathogenesis-related genes in roots. High light, osmotic and salt stresses support the beneficial interaction between the plant and the fungus. *P. indica* reduces stomata closure and H_2O_2 production after *A. brassicae* infection in leaves and suppresses the defense-related accumulation of the phytohormone jasmonic acid. Thus, shifting the growth conditions towards a mild stress promotes the mutualistic interaction, while optimal supply with nutrients or low stress diminishes the benefits for the plant in the symbiosis.

Keywords

Biotic and abiotic stress, defense, mutualism, metal resistance, light stress, osmotic stress, salt stress, biophoton, stomata, reactive oxygen species, phytohormones, root architecture

Abbreviations

P: phosphorus; C: carbon; N: nitrogen; Pi: Inorganic phosphate; JA: jasmonic acid; WT: wild-type; Nsurf: Network Surface Area; Nlen: Network length; SEM: Scanning Electron Microscopy; ROS, reactive oxygen species

Introduction

Biotic and abiotic stresses cause physiological and hormonal imbalances, nutrition deficiency, ion toxicity, reduction of the defense capacity and thus reduce plant performance and agricultural yields. Stresses can be reduced by different strategies including symbiotic interactions. Besides stress tolerance, a symbiotic interaction can promote the biomass production of the plant (Nadeem et. al. 2013) and ensures a better survival in nature. Fungi and microbes profit from a symbiotic interaction by the photoassimilates from the host.

Arbuscular mycorrhiza (AM) fungi play a key role in ecosystems (Helgason and Fitter 2009; Smith and Smith 2011; Smith et al. 2011; Miransari 2010). Inorganic phosphate (Pi) uptake from the soil and carbon transfer from the host to the fungus establish a C-P organic balance which is crucial for symbiotic interactions (Smith and Smith 2011). Pi uptake is energy-consuming for plants and fungi and its uptake by roots results in depletion of the surrounding soil area and consequently requires root growth and development for reaching new areas with enough Pi (Tinker and Nye 2000). Plants respond to Pi limitations by increasing the root hair number and length, Pi mining from unsolvable resources by malic and citric acids or acid

phosphatases (Marschener 1998; Khan et al. 2000) and symbiosis with fungi (Lambers et al. 2008), which can increase the solvability of Pi forms (Khan et al. 2000).

AM fungal mycelium enhances the hydraulic conductivity in roots (Rosendahl and Rosendahl 1991; Giri et al. 2003). Water deficiency affects growth and plant yield (Kramer and Boyer 1997; Feng et al. 2002) *via* modification of the plant osmotic potential (Ruiz-Lozano 2003). The plant itself uses anatomical, physiological, cellular mechanisms (Bray 1997) and symbiosis (Sylvia et al. 1993) to reduce the negative effects. Under drought conditions the AM symbiosis increases plant resistance (Sylvia et al. 1993; Subramanian et al. 1995; Brown and Bethlenfalvay 1988; Subramanian et al. 2006; Augé 2001) by increasing nutrient uptake, enlarging the root surface area (Augé et al. 1994; Subramanian et al. 2006), and adjusting the osmotic potential (Azcón et al. 1996; Goicoechea et al. 1998; Ruiz-Lozano 2003; Ruiz-Lozano et al. 2006). The fungus preserves the moisture by generating glomalin-soil aggregations (Augé 2001; Ruiz-Lozano 2003; Augé et al. 2004; Rillig 2004), while the plant responds to the presence of the fungus by altering the gene expression pattern and physiology (Ruiz-Lozano et al. 2006; Aroca et al. 2008; Boomsma and Vyn 2008), e.g. by higher production of antioxidants (Ruiz-Lozano 2003) or improvement of water movement into the plant (Augé 2001). The water potential is much higher in mycorrhizal colonized relative to non-colonized plants (Subramanian et al. 2006).

Reduction of heavy metal uptake in plants *via* production of metallophytes is another effect of AM fungi interacting with the plant root (Berreck and Haselwandter 2001; Hildebrandt et al. 2007). Different plant species produce metallothionin under heavy metal stress (Rivera-Becerril et al. 2005; Miransari 2010). Hildebrandt et al. (2007) demonstrated that heavy metal uptake into roots is reduced by the fungus-produced glycoprotein glomalin. Glomalin is highly persistent in soil, affects the soil structure and reduces soil density and indirectly improves plant growth, beside its direct effect on heavy metal tolerance (Rillig et al. 2001). Fungi also precipitate metal ions (Vahabi et. al. 2011, 2014; Feng et al. 2013).

Half of the cultivable lands will become saline in the next 40 years (Wang et al. 2003) which will result in increasing levels of hyperionic and hyperosmotic stress (Vaidyanathan et al. 2003; Ramoliya et al. 2006; Porcel et al. 2012) and subsequently in higher oxidative stress (Gill and Tuteja 2010). Production of antioxidant enzymes helps plants to survive better under salt stress conditions (Sekmen et al. 2007; Garg and Manchanda 2009; Turkan and Demiral 2009; Manchanda and Garg 2011; Ruiz-Lozano et al. 2012). Fungi-mediated antioxidants (ascorbic acid, α-tocopherol, glutathione and carotenoids) cause reduction of hydrogen

peroxide and peroxidation of lipids (Evelin and Kapoor 2013). Root dependency of the symbiotic fungi increases under salinity and fungi can alleviate salt stress effects of the plant (Tian et al. 2004). In salty conditions, plant phosphorus (P) and magnesium (Mg^{2+}) levels are increased in the presence of AM fungi by the accumulation of osmolytes in roots, such as carbohydrates and electrolytes (Ruiz-Lozano et al. 1996; Feng et al. 2002; Boomsma and Vyn 2008; Daei et al. 2009). The deficiency of Mg^{2+} in plants results in reduction of chlorophyll formation and causes adverse effects on photosynthesis (Giri et al. 2003). The symbiotic plant-fungi interaction also improves NO_3^-, Ca^{2+} and K^+ uptake under salt stress (Vicente-Sánchez et. al. 2014).

In this study we analyze the *Piriformospora indica*/Arabidopsis interaction by manipulating stress conditions during their co-cultivation. The beneficial interaction of the root endophyte *P. indica* with a multitude of horticulturally and agriculturally important plants as well as model plants such as Arabidopsis leads to growth promotion, increased biomass production and enhanced resistance/tolerance against biotic and abiotic stress (cf. Varma et al. 1999; Pham et al. 2004; Peškan-Berghöfer et al. 2004; Sherameti et al. 2005; Waller et al. 2005; Shahollari et al. 2007; Vadassery et al. 2009; Sun et al. 2010; Lee et al. 2011; Johnson et al. 2011). The fungus can be cultivated axenically on synthetic or complex media without a host (Varma et al. 1999, 2001; Peškan-Berghöfer et al. 2004). Once inside the roots, the fungus gets access to photoassimilates and other plant nutrients, which further promotes colonization and proliferation (Sherameti et al. 2005; Yadav et al. 2010).We demonstrate that a mild increase in various unrelated stresses strengthens the symbiotic interaction of *P. indica* with Arabidopsis, which results in better performance of the plant.

Results

Nutrient availability in Arabidopsis/*P. indica* interaction

We first established co-cultivation conditions in which the two symbionts have different access to nutrients from the PNM medium. Arabidopsis seedlings co-cultivated with *P. indica* in a PNM aquaculture for 30 days accumulate less biomass compared to seedlings grown without the fungus (Fig. 1A, B). Growth of the seedlings was also reduced when the two symbionts were co-cultivated on solified PNM medium in Petri dishes. Separation of the biological material from the PNM medium by a nylon membrane restricted the access of the roots and hyphae to the nutrients. Under these conditions, the presence of the fungus is favorable for plant growth which results in a slight, but significant increase in the plant biomass, relative to growth without the fungus. Further restriction of the access to nutrients is

achieved by a cellophane membrane which cannot be passed by roots and fungal hyphae. This resulted in an even stronger growth promotion of the plant in the presence of the fungus, relative to the seedlings grown without the fungus (Fig. 1A, B). This suggests that limited access to the PNM nutrients is crucial for the stimulatory effect of the fungus on plant biomass production.

The *P. indica*-induced changes in plant growth are reflected by the fungus-induced alterations in the root architecture. It is obvious that root growth stimulation by *P. indica* increases the more the access to the medium is restricted (aquaculture < agar < membrane < cellophane). Using the software program developed by Galkovskyi et al. (2012), the surface area (Nsurf), length (Nlen), perimeter (Perim) and volume of the root system was quantified for the four growth conditions with and without the fungus (Fig. 2A). They confirm an increase in root growth stimulation by *P. indica* with decrease in accessibility to the nutrients in the medium. The most dramatic increase was observed for roots which were co-cultivated with *P. indica* on the cellophane membrane (Fig. 2B, C). While barely any lateral roots can be detected in the uncolonised control, lateral root development is strongly promoted by *P. indica*.

Furthermore, physical contact between the two symbionts strongly stimulates *P. indica*-mediated growth promotion of Arabidopsis seedlings. When the fungus grows under the cellophane membrane (Fig. S1) no physical contact to the host can be established. Under this condition, the growth-promoting effect of the fungus on the seedlings was more than 10-times lower compared to co-cultivation conditions on the surface of the cellophane membrane (Table S1). We conclude that a physical contact cannot be replaced by chemical communication between the two symbionts. In addition, restriction in nutrient access strengthens the physical contact between the two symbionts: Scanning electron microscopy (SEM) (Fig. 2C) shows the mycelial network when the two symbionts are in physical contact. The fungal mycelium grows on the root surface, covers the surrounding area of the roots and penetrates into the root epidermal cells. The root-associated mycelial network extends to distantly located cellophane areas. The capillary activity from the root cells is supported by this fungal network, which allows a more efficient nutrient and water uptake from the accessible medium, compared to roots growing without the fungus.

To further support the concept that limitations in nutrient availability promotes plant growth by *P. indica*, Arabidopsis seedlings with and without the fungus were grown on different PNM concentrations (0.25x; 1x; 4x). The strongest growth-promoting effect of the fungus was observed on 0.25x PNM medium and the smallest effect on 4x PNM medium (Fig. 3A).

Microscopic analyses and determination of the ratio of fungal *ITS* mRNA/plant *GAPDH* mRNA (Jacobs et al. 2011) demonstrate that root colonization is higher on low PNM medium and reduced of high PNM medium (Fig. 3B, C). This suggests that root colonization increases with decrease in nutrient availability.

Lower nutrient availability resulted also in the down-regulation and higher nutrient availability in the up-regulation of the pathogenesis-related genes *PR1*, *PR2*, *PR4* and *PR5* in shoots indicating a general repression of the plant defense system (Fig. 3D). This might be caused by less resource availability for the plant defense machinery under nutrient-limiting growth conditions, or higher root colonization results in a more efficient down-regulation of the plant defense machinery.

In conclusion, lower nutrient availability promotes the symbiotic interaction. The role of the *P. indica* in promoting the plant´s excess to the nutrients is manifested by higher root colonization and root and plant growth stimulation. On the other hand, the plant´s investment in defense is reduced under nutrient-limiting conditions.

Role of Pi, NO_3^- and SO_4^{2-} in the *P. indica*/Arabidopsis interaction

Pi availability is a classical model to study the role of beneficial fungi in helping the plant to obtain better access to this growth-limiting nutrient. Several studies have demonstrated that Pi limitation restricts Arabidopsis growth and promotes the Arabidopsis/*P. indica* interaction (Vadassery et al. 2008; Bakshi and Oelmüller, personal communication). Under our growth conditions, the shoot fresh weight of *P. indica*-colonized seedlings grown on full PNM medium on the nylon membrane is increased by 31 ± 3% compared to the uncolonized control (Fig. 4B). If the KH_2PO_4 concentration in the PNM medium is reduced 10-fold, the growth-stimulating effect increased to 58 ± 4% (Fig. 4B). Similar results were also obtained for other ions: 10-fold reduction of the KNO_3 concentration leads to an increase in the fresh weight from 31 ± 3% to 46 ± 2%, and of the $MgSO_4$ concentration from 31 ± 3% to 54 ± 5% (Fig. 4B). These examples support the concept that limitations in Pi and other essential ions promote the benefits for the plant in the interaction.

Heavy metal and osmotic stress in Arabidopsis/*P. indica* interaction

To further support the idea that mild stress conditions promote the *P. indica*/Arabidopsis interaction, we exposed colonized and control seedlings to various heavy metal and osmotic stresses.

As expected, exposure of colonized and uncolonized Arabidopsis seedlings to heavy metal stress severely reduced their shoot fresh weights. However, when the % increase in shoot weight of colonized *versus* uncolonized seedlings is considered, the colonized seedlings perform better than the uncolonized controls (Fig. 4A). It appears that the benefits for the plants induced by the fungus increase with increasing toxicity of the metal ions (Zn < Ag = Ni < Cr = Co = Cd).

Similar effects were observed for seedlings exposed to osmotic stress, i.e. PNM medium plus 200 mM NaCl or 50 mM mannitol or 10% PEG. The strongest effect was observed for PEG-exposed seedlings (Fig. 4C).

These examples demonstrate that the seedlings profit more from the symbiosis when they are exposed to a mild heavy metal or osmotic stress.

Light intensity in Arabidopsis/*P. indica* interaction

While minerals are taken up from the soil, light stress acts directly on leaves. Since plant performance is strongly affected by light quality and intensity, we tested whether root colonization by *P. indica* has an effect on light stress to the leaves.

P. indica-colonized and control plants were grown under 25, 50 and 110 µmol m^{-2} sec^{-1} continuous illumination from the top. Figure 5A demonstrates that the increase in the light intensity results in reduced shoot biomass of uncolonized seedlings, while the opposite is observed for colonized seedlings. This demonstrates that continuous illumination with higher light intensities is stressful for the seedlings, and that root colonization counteracts this stress by root-to-shoot signaling.

To gain more inside into these processes, we measured fluorescence parameters for the efficiency of the photosynthetic electron flow under the different conditions. The photosynthetic yield represented by the Fs/Fm parameters in low light (25 and 50 µmol m^{-2} sec^{-1}) is higher in uncolonized than in colonized plants. The opposite effect was observed for high light-grown plants (110 µmol m^{-2} sec^{-1}). Consistent with these observations, the NPQ values representing energy loss by heat dissipation gave the opposite results (Fig. 5B). The data demonstrate that the efficiency of the photosynthetic electron flow decrease gradually with increasing light intensity in uncolonized plants, and that fungal signals from the roots counteract the efficiency loss (Fig. 5B).

Pathogenic fungi in Arabidopsis/*P. indica* interaction

Finally, we exposed Arabidopsis leaves to a mild dosis of the pathogenic fungus *Alternaria brassicae* (Fig. 6) and tested whether this has an effect on *P. indica*-induced growth promotion. Fig. 6A demonstrates that growth promotion by the beneficial fungus was stronger in pathogen-exposed seedlings when compared to seedlings which were not treated with *A. brassicae*. Infection of the leaves with *A. brassicae* induced H_2O_2 production, and seedlings which were co-cultivated with *P. indica* produced significantly less H_2O_2 (Fig. 6B). Similarly, *A. brassicae* infection to the leaves resulted in stomata closure and this effect was also reduced in *P. indica*-exposed seedlings (Fig. 6C). The latter two observations suggest an efficient root-to-shoot communication.

Biophotons are ultra-weak emission of photons which mainly resulted from the generation of reactive oxygen species (ROS) and metabolic activities as by-products of cellular respiration (Takeda et al. 2004; Kobayashi et al. 2014). Biophoton measurements have been used for demonstration of stress conditions in various organisms (Takeda et al. 2004; Wijk et al. 2013; Kobayashi et al. 2014). Biophoton emission after *A. brassicae* infection was lower in *P. indica*-colonized seedlings relative to the seedlings not exposed to *P. indica*. This confirms that *P. indica* reduces the stress induced by *A. brassicae* infection (Fig. 6D).

The phytohormones jasmonic acid (JA), JA-isoleucine, and oxophytodinoic acid are crucial for the activation of defense responses. Figure 7 demonstrates that JA, JA-isoleucine, and oxophytodinoic acid accumulate in the shoots of Arabidopsis seedlings after leaf-infection with the pathogenic fungus *A. brassicae*. Pre-treatment of the seedlings with *P. indica* reduced significantly hormone accumulation. We conclude that *P. indica* represses Alternaria-induced phytohormone accumulation in Arabidopsis shoots.

Taken together, increased abiotic and biotic stress results in the promotion of plant biomass and performance by *P. indica*.

Discussion

We demonstrate that a mild increase in quite different stresses promotes the symbiotic interaction between *P. indica* and Arabidopsis roots, which ultimately results in better plant performance. Our experimental setup included abiotic and biotic stress experiments, both to roots and shoots, in which the two symbionts had access to different nutrient concentrations, were exposed to heavy metals with different toxicities, to different osmotic and light stress conditions and a pathogen. We measured different well characterized responses of the plants to these stresses which describe the fitness of the plant in the presence and absence of root

colonization by *P. indica*. We optimized the conditions such that the highest applied stress reduced plant performance but did not cause irreversible damage. All experiments demonstrate that the fungal effect on plant growth and performance increases with an increase in the applied stress. These experiments also highlight the importance of the experimental setup for studying plant/microbe interaction. For instance, co-cultivation of the two symbionts on PNM medium does not result in growth promotion, unless access to nutrients in the PNM medium is restricted by a nylon or cellophane membrane.

Limited access to or availability of nutrients strengthens the symbiotic interaction which also results in the stimulation of root growth and severe alterations in the root architecture. Similar results for the *P. indica*/Arabidopsis interaction were obtained when specific ion (Pi, NO_3^-, SO_4^{2-}) concentrations were reduced in the PNM medium. Likewise, AM symbiosis is stimulated by phosphorus limitation and contributes to phosphorous and nitrogen acquisition (Bonneau et al. 2013, and references therein). A number of studies have demonstrated that mycorrhizal fungi and plant growth promoting rhizobacteria improve crop productivity under stressful environments (Nadeem et al. 2014, and references therein). In AM symbiosis, a more intense interaction of the two partners under nutrient-limiting conditions has been intensively studied for Pi^- (Bolan et al. 1987), NO_3^- (Jin et al. 2012) and SO_4^{2-} (Gahan and Schmalenberger 2014). Smith and Smith (2012) proposed that the level of colonization is crucial for a beneficial interaction in AM symbiosis, and in Pi^- and NO_3^- rich soil the level is lower in AM plants. This results in growth depression because the beneficial traits in the symbiosis decrease. In media with low PNM concentration the plant is more dependent on the fungus. The higher fungal RNA/plant RNA ratio in the colonized roots of seedlings grown under low PNM medium indicates that the roots are better colonized for acquisition of nutrient for plant growth. Down-regulation of *PR* gene expression in low PNM medium provides optimal condition for colonization by *P. indica*. Conversely, in higher nutrient concentration, the plant does not need the fungus for nutrient aquisition and root colonization is reduced. Up-regulation of the *PR* genes and induction of the plant defense system under these conditions shows that the plant produces anti-fungal compounds to restrict or even prevent root colonization. Thus, the level of stress is defining the level of biotic interaction between the two symbionts. Under nutrient-limiting conditions, the fungus stimulates root growth more than under conditions with sufficient nutrient supply, as shown by the Nsurf, Nlen and the root surface volume values (Fig. 2A).

We also demonstrate that heavy metal tolerance of Arabidopsis seedlings is stimulated by *P. indica*, and the protective role of the fungus increases with increasing toxicity of the heavy metal ion. Similarly, plants in mycorrhizal associations are less sensitive to heavy metal stress than non-mycorrhizal plants (Schützendübel and Polle 2002), and cadmium-induced changes in mycorrhizal roots were absent or smaller than those in non-mycorrhizal roots (Repetto et al. 2003). Several mechanisms can account for these observations. The heavy metals could accumulate primarily in the fungal hyphae and thus to a lesser extent in the roots. Alternatively, metal chelators or organic acids could be secreted by fungi or mycorrhizal roots (Khan et al. 2000; Ahonen-Jonnarth et al. 2000). The antioxidant activity becomes activated after exposure to heavy metal stress and participates in protecting the roots against this stress (Abdel Latef 2011). Detoxification of heavy metals by fungi occurs also through the generation of nanoparticles, extracellular particles, which scavenge the metal ions and reduces their concentrations prior to the up-take of the roots (Feng et al. 2013; Vahabi et al. 2011). Considering that these detoxification mechanisms do not distinguish between different heavy metal ions, it is reasonable to assume that detoxification of highly toxic ions has a stronger effect for the plant than detoxification of less toxic heavy metals. Taken together, better plant growth and performance include also *P. indica*-mediated detoxification or sedimentation of toxic metal cations outside or inside of plant and fungal cells.

Similar results were obtained when Arabidopsis seedlings were exposed to osmotic stress. The growth-promoting effect of *P. indica* increased with increasing osmotic stress. Also Augé et al. (2014) studied resistance of mycorrhizal plant to NaCl stress. They found that the efficiency of mycorrhiza on plant perfomance depends on the stress severity.

Finally, we applied two types of stress to the leaves and checked how they are affected by signals from colonized roots: light and infection with Alternaria spores. With increase in light intensity, colonized plants perform better compared to those grown under low light conditions. Under high light conditions, the ROS level in the leaves increase, and ROS is also produced after Alternaria infection. The ROS levels in the leaves are reduced in *P. indica*-colonized plants. This might be caused by the stimulation of the antioxidant capacity in the plant (Baltruschat et al. 2008; Vadassery et al. 2009) to detoxify the high ROS levels. Furthermore, the root-associated fungus promotes the performance of the aerial part by signals from the roots. We introduced the new and highly sensitive biophoton technology, which detects ultra-weak emission of photons mainly from the generation of ROS and metabolic activities as by-products of cellular respiration (Takeda et al. 2004; Kobayashi et al.

2014). Biophoton measurements represent the amount of ROS in the cell and have been used for demonstration of stress condition (Takeda et al. 2004; Wijk et al. 2013; Kobayashi et al. 2014). We demonstrate that this technology is appropriate to quantitatively describe stress in plants, and is thus a reliable tool to measure plant fitness under different physiological conditions. We demonstrate that this technology is able to measure differences in *P. indica*-colonized and uncolonized plants exposed to low stress.

JA, JA-isoleucine, and oxophytodinoic acid are involved in the activation of plant defense against biotic stresses (Sun et al. 2014). Infection of Arabidopsis leaves with *A. brassicae* spores results in accumulation of these hormones in shoots in response to infection. The pathogen-induced phytohormone accumulation in leaves is suppressed by the beneficial fungus *P. indica* when roots are pre-treated with this fungus. A comparable systemic effect was also shown for Arabidopsis leaves infected with *Verticillium dahliae* (Sun et al. 2014).

Collectively, these results demonstrate that the beneficial effects of *P. indica* require stress. Increase in stress within a reasonable and non-toxic scale stimulates the symbiotic interaction which in turn results in better performance of the plant.

Materials and methods

Co-cultivation of *A. thaliana* with *P. indica* in different conditions

A modified Kaefer medium (KM; Hill and Kaefer 2001) was used for the propagation of *P. indica* on a Petri dish (Johnson et al. 2011). After growing for 14 days at room temperature, a *P. indica* plaque with 5 mm diameter (Control: plaque without hyphae) was placed on PNM media with a nylon membrane (pore size 70 µm) on the top. The fungal culture was kept at the same condition for 1 week until the *P. indica* mycelium covered the entire membrane surface. For co-cultivation, 12 days-old seedlings of *Arabidopsis thaliana* were grown on MS media as described in Johnson et al. (2011). Four Arabidopsis seedlings were transferred to the plates colonized by *P. indica* as well as to control plates and grown for 10 days in long day condition [55 (± 10) µmol m^{-2} sec^{-1} light from the top]. Different concentrations of PNM (0.25x, 1x and 4x) were used to study the nutrition effect on the *P. indica*/Arabidopsis interaction. To investigate the effect of N, P and S, the respective salts in the PNM medium were reduced to the final concentrations of 0.5 mM, 0.25 mM and 0.125 mM for KNO_3, $MgSO_4$ and KH_2PO_4, respectively. Continuous light of 12.5 ± 2.5, 50 ± 10 and 110 ± 10 µmol m^{-2} sec^{-1} was given from the top to analyze the effect of the light intensity on the symbiotic interaction. Biotic stress was investigated with the pathogenic fungus *Alternaria brassicae*.

After infection of Arabidopsis seedlings co-cultivated with or without *P. indica* for 7 days, the leaves were infected with a spore solution. *A. brassicae* was cultured on PDA media for two weeks at room condition. The spores were collected with a sterile scalpel and three times washed with distilled sterile water. Spore concentration was adjusted to the $1x10^6$ spore/ml and 2 µl of this suspension was used for leaf infection. As control, sterile water was used instead of the *A. brassicae* inoculum.

Nutrition availability study

Sterilized Arabidopsis seeds were placed on PNM plates with different nutrition concentrations, and inoculated with 2 µl of the *P. indica* spore solution (1×10^6 spore/ml). Distilled water was used as control and the seedlings were kept for 2 days at 4°C before transfer to the respective light conditions for four weeks under long day condition (18 h light and 6 h dark) and a light intensity of 55 ± 10 µmol m^{-2} sec^{-1}, applied from the top. Different access to nutrient supply was achieved by growing the symbionts under four different conditions: an aquaculture system (Vahabi et al. 2014), growth of the symbionts on solid PNM media, on solid PNM media covered with a nylon membrane, or on solid PNM media covered with a cellophane membrane.

Co-cultivation of the symbionts under aquaculture condition was performed in Magenta boxes with 300 ml sterile PNM liquid media. The boxes were covered by aluminum foil and a microtube from which the tip was removed, filled with solid PNM media placed in a hole in the middle of the aluminum foil as described in Vahabi et al. (2014). For solid PNM media, 300 ml PNM media containing 1% agar in a Magenta box was used. A 70 µm pore-size nylon membrane was placed on top of 300 ml PNM medium solidified with agar. For the co-cultivation conditions with cellophane, the nylon membrane was replaced by a cellophane membrane.

Root architecture

Root architecture studies were performed by co-cultivation of Arabidopsis seedlings with *P. indica* in vertical square plates. 12 days-old seedlings grown on MS media were transferred to the PNM media prepared with gelrite and kept vertically for 10 days under long day conditions (65 µmol m^{-2} sec^{-1}, light from the top). *P. indica* inoculation was done at the same time (Johnson et al. 2011) by placing 2 agar plaques of 14 day-old fungus in KM media to the center of the square plates, in 5 cm distance to each other and 2.5 cm distance from the edge of plates.

To study how the root hair architecture of the seedlings is affected by *P. indica*, Arabidopsis seeds were placed on the surface of PNM media covered by a cellophane membrane together with *P. indica* spores. After 4°C for 48 h, they were kept under long day condition for three weeks. 2 µl of a *P. indica* spore solution (as described above) was used for inoculation of the seeds on the surface of the cellophane membrane. Control seeds were treated with sterile water instead of the *P. indica* inoculum. The GiA Roots software was used to analyze the differences in the root architecture between control and *P. indica*-treated plants according to Galkovskyi et al. (2012).

For more detailed analyses of the root architecture the Network surface area parameter (Nsurf, the sum of the pixel of the local surface area of root or root hairs from the image), the Network area parameter (the number of root or root hair pixels in the image), the Network length parameter (Nlen, the total number of pixels in the network skeleton), the Perimeter parameter (Perim, the total number of root or root hair pixels connected to a background pixel), the Network volume (the sum of the root or root hair volume in pixel as approximated by a tubular shape whose radius was estimated from the image) were calculated as described by Galkovskyi et al. (2012).

To analyze the relevance of a physical contact of the roots with *P. indica*, the two symbionts (Arabidopsis seedlings and 2 µl of a *P. indica* spore suspension) were co-cultivated on a cellophane membrane as described above and compared with conditions in which 2 µl of the spore suspension of *P. indica* was applied under the cellophane membrane. Microscopic and PCR analyses confirmed that the two symbionts did not have physical contact when they are growing on opposite sides of the cellophane membrane. The seedlings were harvested after 3 weeks of co-cultivation, and the fresh weights of seedlings grown on the membrane alone and with *P. indica* on the same or opposite sides of the membrane were compared.

Light stress, fluorescence parameters

Co-cultivation of the two symbionts was performed as described above on PNM medium, except that the plates were exposed to continuous illuminations with different light intensities (25 ± 5 µE, 50 ± 15 µE and 110 ± 10 µE) for 10 days. The fluorescence parameters were measured with a FluorCam 700MF instrument and analysed with the Flucam 5.0 software. The data are averages for 30 seedlings and 3 independent biological experiments.

Heavy metal experiments

Arabidopsis seedlings were co-cultivated with *P. indica* (or mock-treated) for 7 days on PNM medium supplemented with $NiCl_2$, $CrSO_4$, $CdCl_2$, Co-acetate, $ZnNO_3$ or $AgNO_3$ (concentrations are given in the Result section) and compared with the PNM control.

Osmotic and salt stress

12 day-old seedling grown on MS media were transferred to the PNM plates containing either NaCl (200mM), PEG (10%) or mannitol (50 mM). These concentrations correspond to osmotic potential of -0.78, -1.24 and -1.69 MPa, respectively (Almansouri et al. 2000). The seedlings were inoculated with a *P. indica* plaque (control: KM medium without the fungus) for one week before the fresh weights of the seedlings were determined.

Biophoton measurement

Biophoton investigation of Arabidopsis leaves was performed with a Zeiss LSM 710 microscope in darkness with the ZEN-software and without laser excitation of the samples. Detached leaves on the microscope glass slides in 100 µl glycerol (50%) matrix were analyzed using a 10x magnification objective (EC Plan-Neofluar 10x/0.30 M27) and an image scanning area of 512 x 512 pixel. The image acquisition setup was selected for three different dyes *via* a smart setup button, and the biofluorescence of the leaves was measured from 382 to 596 nm**.** To minimize the crosstalk among each fluorophore, the "Best signal mode" sequential scanning was chosen. The emission detection range of each emission filter had been modified around the maximum of the filters to a 20 nm spectral range. Due to the weak autofluoresence of the leaves, the pinhole of each channel was adjusted to the maximum value, to collect more light emission of the leaves by the detector. The voltage on the photomultiplier tube which detects the emitted light from the sample was set to 800 to increase the PMT sensitivity to obtain optimal intensity and background signals. Grays scale was used for image processing of the 512 × 512 pixel area by the ImageJ 1.48v software and the mean gray value was used for comparison of relative amounts of biophotons recorded on the images. Background noises induced by high-energy particles were recorded by imaging of biophoton blank samples and their mean gray values were subtracted from all sample measurements. The results show an average of images recorded from 30 leaves of 3 independent biological experiments.

RNA analysis

RNA was isolated from shoots by adding 1 ml Trizol to 100 mg tissue powdered in liquid nitrogen and shacking for 10 min in a microtube. After the addition of 200 µl chloroform, the

tubes were shaken for 10 more min and centrifuged for 30 min at 10.000 rpm at 4°C. The supernatant was transferred to a new microtube and mixed with half volume of ice-cold isopropanol and extraction buffer (0.8 M Na-citrate and 1.2 M NaCl). After 10 min incubation at room temperature, followed by centrifugation for 30 min at 11.000 rpm at 4°C, the pellet was washed with 1 ml 70% ethanol, dried and dissolved in 50 µl distilled H_2O. Oligo dT primers were used for reverse transcription of 1 µg of total RNA with the Qiagen kit (Omniscript, Qiagen, Hilden, Germany). RT-PCR was conducted with the primer pairs given in Table S1. Arabidopsis *GAPDH* and *P. indica ITS* were used as housekeeping genes for the two organisms.

The "Bio-Rad CFX connect real-time system" and "Bio-Rad CFX manager version 3.1" (Bio-Rad, Munich, Germany) were used for preforming real-time quantitative RT-PCR with Taq DNA polymerase and Eva green (Bio-Rad) in a final volume of 20 µl for the amplification of the PCR products. The reactions were performed with 95°C 2 min, 39× (95°C 30 s, 60°C 40 s, 72°C 45 s), 72°C 8 min followed by a melting curve program (55-95°C in increasing steps of 0.5°C). Annealing temperature was adjusted according to the primers. Three amplification repetitions for at least three independently isolated RNAs were used for all biological repetitions. Bio-Rad CFX Manager software was used for fold induction value calculations.

H_2O_2 measurements and ROS staining

Arabidopsis seedlings co-cultivated with *P. indica* for 15 days were infected with *A. brassica*e spores. After 10 days, they were stained with 3,3'-diaminobenzidine (DAB) as described by Daudi et al. (2012). As a result of staining a brown precipitate upon oxidation was formed, which is insoluble in aqueous and organic solvents (Gallyas et al. 1982; Vidossich et al. 2012). For the detection/quantification of H_2O_2 inside the plant material, 100 mg of stained tissue was washed with acetone three times, ground to a fine powder and - after drying - dissolved in 1 ml DMSO at 90°C for 1 h. The supernatant was separated from the precipitate by centrifugation at 10,000 rpm for 5 min and further used for spectrophotometric measurements at 270 nm (Perkin Elmer, Lambda 12) as described by Greenfield et al. (2010). The poly-DAB concentration of the plant tissue was correlated to the H_2O_2 concentration using a standard curve which was generated by the application of four different concentrations of H_2O_2 (0.1, 1, 10, 100 µg).

Microscopy of roots and stomata

Fluorescent staining of root samples was performed with fuchsin acid and trypan blue and the material was analysed with a Zeiss fluorescent microscope Oxiovert (Vahabi et al. 2011).

Stomata staining was done according to Vahabi et al. (2015). The data are averages of 20 leaves from 10 seedlings with 3 independent biological repetitions.

Plant hormone measurements

10 day after inoculation of control and *P. indica* treated seedling with *A. brasicae*, the shoots were used for quantification of JA, JA-isoleucine and oxophytodinoic acid. The phytohormone levels are expressed relative to the dry weight according to Sun et al. (2014).

Scanning Electron Microscopy analysis

For SEM analysis, samples were fixed overnight with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.0). They were dehydrated with ethanol in serially increased concentration, followed by critical point drying in a Leica EM CPD300 Automated Critical Point Dryer (Leica, Wetzlar, Germany). The samples were coated with carbon (20 nm) in a BAL-TEC SCD005 Sputter Coater (BAL-TEC, Liechtenstein) and analyzed at different magnifications in a LEO 1530 Gemini field emission scanning electron microscope (Zeiss, Oberkochen, Germany) at 12 kV acceleration voltage and a working distance of 6 mm using an inlense secondary electron detector and a scintillation type backscatter electron detector (Centaurus detector, K.E. Developments, Cambridge, UK).

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Supplementary Materials

Figure S1. Scheme for co-cultivation of Arabidopsis seedlings with *P. indica* grown under or on top of a cellophane membrane.

Table S1. List of primers used for Real-time PCR.

Conflict of interests

The authors declare that they have no conflict of interest.

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Figures and Tables

Figure 1. *A. thaliana* seedlings grown under long day light condition (65 µmol m^{-2} sec^{-1}) and different access to the nutrients in PNM medium (aquaculture, agar, membrane or cellophane) with or without *P. indica*. **A.** Typical pictures of the seedlings after 30 days of co-cultivation under the four different conditions. **B.** Shoot fresh weights of 30 day-old seedlings shown in A. **C.** Weight promotion of the shoots by *P. indica* (in %) relative to the uncolonized control. Results are based on 3 independent experiments with 10 individual seedlings each. Bars represent SE. Asterisks indicate significant differences (relative to its own control) as determined by T-test (** $P \leq 0.01$; *** $P \leq 0.001$).

Figure 2. Effect of *P. indica* on the root architecture of *A. thaliana* seedlings grown for 12 days on PNM media under long day light condition (65 µmol m^{-2} sec^{-1}). **A**. Root architecture of Arabidopsis seedlings grown on PNM media with and without cellophane. **B**. Microscopical view of the roots' network in control and *P. indica*-treated seedlings (+ *P. indica*) grown on PNM plates covered with cellophane. Pictures were from the GiaRoot software. **C.** SEM images of control and *P. indica*-colonized Arabidopsis roots after 10 days of colonization. All results are based on 3 independent experiments with 10 individual seedlings each. Bars represent SE. Asterisks indicate significant differences (relative to its own control) as determined by T-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Figure 3. Effect of *P. indica* on *A. thaliana* shoot growth (**A**), root colonization (**B** and **C**), and expression of defense genes (**D**) in different PNM concentration [7 days co-cultivation under long day light (50 ± 15 µmol m^{-2} sec^{-1})]. **A**. Shoot fresh weight (*left*) and promotion in % relative to the uncolonized control (*right*). **B**. Fluorescent microscopy of Arabidopsis roots stained with trypan blue and fuchsin acid. **C.** *P. indica ITS* mRNA level (relative to plant *GAPDH* mRNA level) from colonized roots grown on different PNM concentrations. **D**. Fold changes of *PR* genes (+ *P. indica* / - *P. indica*) in Arabidopsis shoots grown on different PNM concentrations. All results are based on 3 independent experiments with 10 individual seedlings each. Bars represent SE. Asterisks indicate significant differences (relative to its own control) as determined by T-test (* $P \leq 0.05$; ** $P \leq 0.01$).

Figure 4. Effect of heavy metals and salts on *A. thaliana* growth in the presence/absence of *P. indica* under long day light (50 ± 15 µmol m^{-2} sec^{-1}). **A.** Shoot fresh weights (*left*) and % weight promotion by *P. indica* (relative to the uncolonized control) (*right*) after 10 days of co-cultivation or mock-treatment. 1 mM of different metal salts was used. **B.** Shoot fresh weights

(*left*) and % weight promotion by *P. indica* (*right*) of seedlings grown for 10 days with/without *P. indica* on different salt concentrations. **C.** Shoot fresh weights (*left*) and % weight promotion (*right*) of 10-day old seedlings co-cultivated with/without *P. indica* under different osmotic and salt conditions. All results are based on 3 independent experiments with 10 individual seedlings each. Bars represent SE. Asterisks indicate significant differences (relative to its own control) as determined by T-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Figure 5. Effect of different light intensities on *A. thaliana* growth in the presence/absence of *P. indica*. **A.** Shoot fresh weights (*left*) and % weight promotion by *P. indica* (*right*) under continuous light and different light intensities (25-110 µmol m^{-2} sec^{-1}). Seedlings were grown for 10 days with/without *P. indica*. **B.** Fluorescence parameters [Y (effective quantum yield of photosystem II), Fs/Fm and NPQ] in Arabidopsis shoots after 10 days co-cultivation with *P. indica* under continuous light (25 ± 5, 50 ± 15 and 110 ± 10 µmol m^{-2} sec^{-1}). All results are based on 3 independent experiments with 10 individual seedlings. Bars represent SE. Asterisks indicate significant differences (relative to its own control) as determined by T-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Figure 6. A. Shoot fresh weights (*left*) and % weight promotion by *P. indica* (*right*) in *A. thaliana* leaves of seedling with/without *P. indica* and 10 days after leaf infection with *A. brassicae*. The seedlings were grown under long day light condition (50.0 ± 15.0 µmol m^{-2} sec^{-1}). **B.** H_2O_2 levels in leaves of Arabidopsis seedlings after infection with *A. brassicae* in the presence/absence of *P. indica* after 10 days. **C.** Stomata closure after Alternaria leaf infection of colonized and uncolonized seedlings after 10 days. **D.** Relative gray values of the images (biophoton records) of Alternaria-infected Arabidopsis leaves treated with/without *P. indica*. All results are based on 3 independent experiments with 10 individual seedlings each. Bars represent SE. Asterisks indicate significant differences (relative to its own control) as determined by T-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Figure 7. JA, JA-Ile and oxophytodinoic acid (OPDA) levels in the shoots with/without *P. indica* and 10 days after leaf infection with *A. brassicae*. The seedlings were grown under long day light condition (50.0 ± 15.0 µmol m^{-2} sec^{-1}). All results are based on 3 independent experiments with 10 individual seedlings each. Bars represent SE. Asterisks indicate significant differences (relative to its own control) as determined by T-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Figure

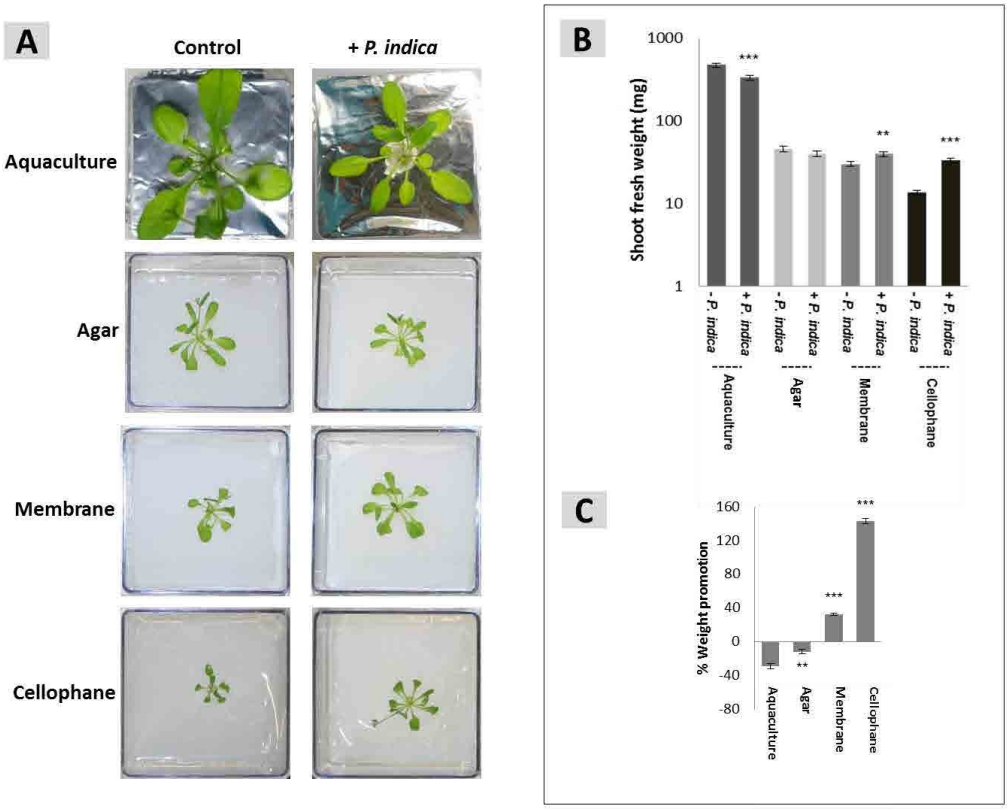
A
Control
+ P. indica
Aquaculture
Agar
Membrane
Cellophane
B
Shoot fresh weight (mg)
1000
100
10
1
***
**
***
- P. indica
+ P. indica
Aquaculture
Agar
Membrane
Cellophane
C
% Weight promotion
160
120
80
40
0
-40
-80
***
**
***
Aquaculture
Agar
Membrane
Cellophane

Fig. 1

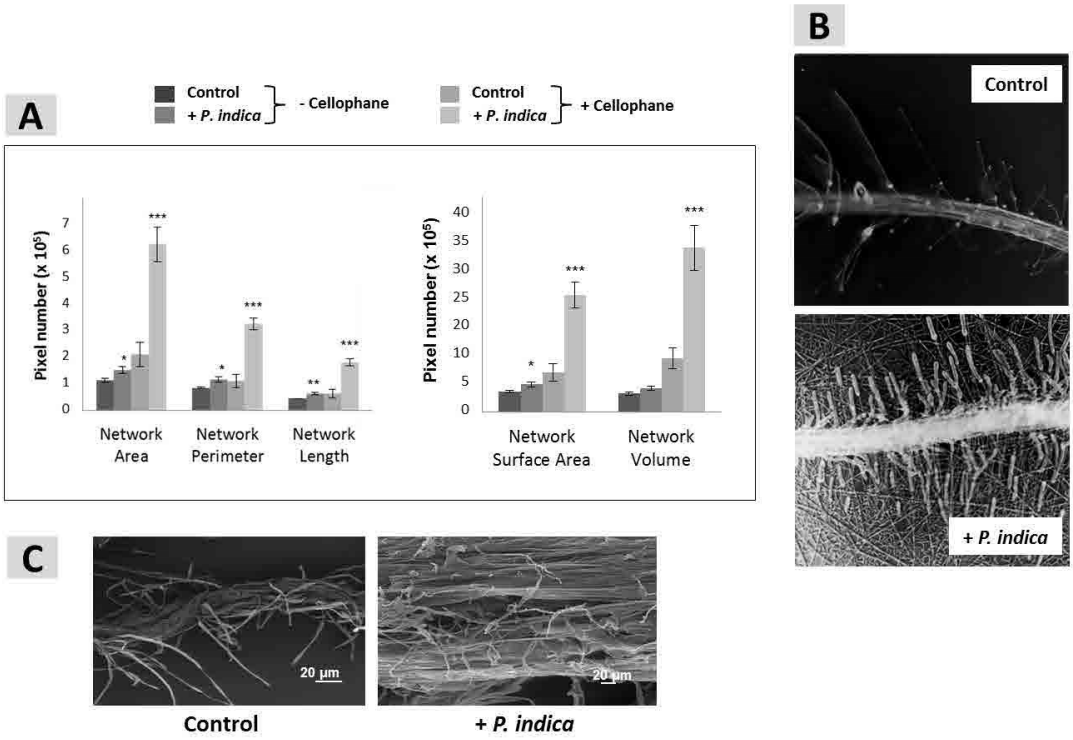
A
Control
+ P. indica
- Cellophane
Control
+ P. indica
+ Cellophane
Pixel number (x 10⁵)
Network Area
Network Perimeter
Network Length
Network Surface Area
Network Volume
B
Control
+ P. indica
C
20 µm
20 µm
Control
+ P. indica

Fig. 2

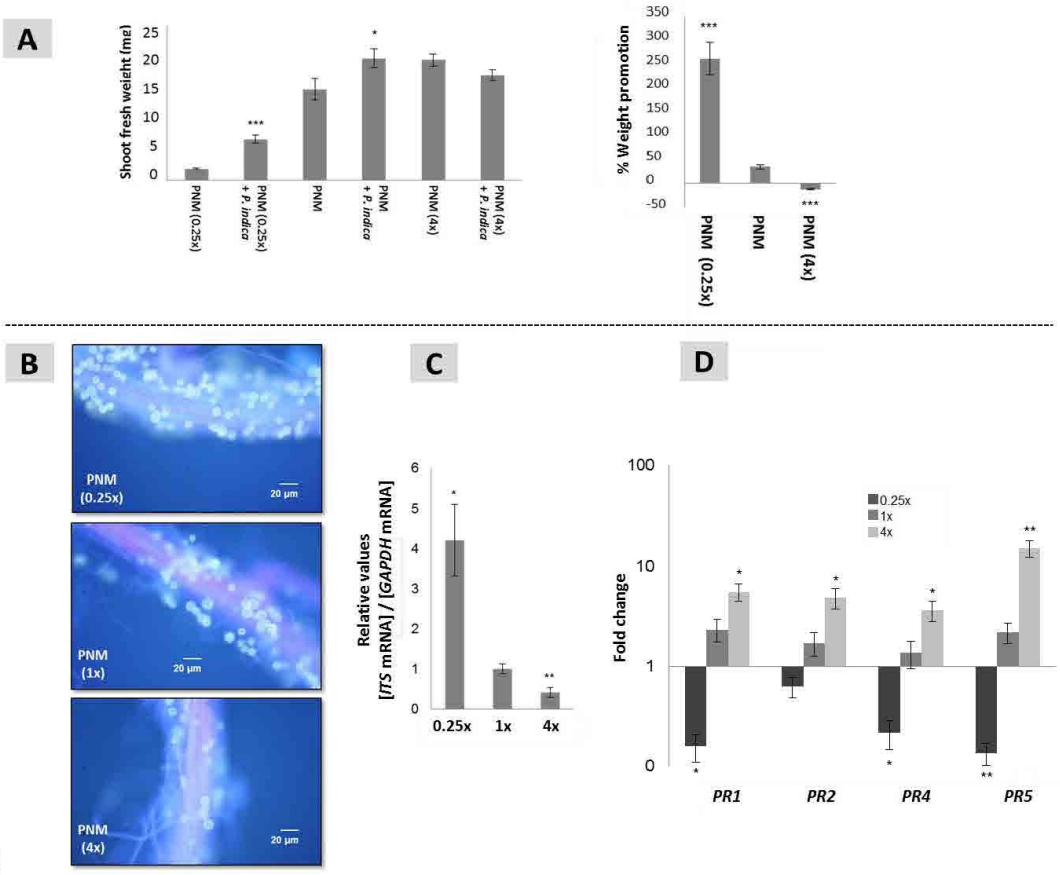
A
Shoot fresh weight (mg)
25
20
15
10
5
0
PNM (0.25x)
PNM (0.25x) + P. indica
PNM
PNM + P. indica
PNM (4x)
PNM (4x) + P. indica
% Weight promotion
350
300
250
200
150
100
50
0
-50
PNM (0.25x)
PNM
PNM (4x)
B
PNM (0.25x)
PNM (1x)
PNM (4x)
20 µm
C
Relative values [ITS mRNA] / [GAPDH mRNA]
6
5
4
3
2
1
0
0.25x
1x
4x
D
Fold change
100
10
1
0
0.25x
1x
4x
PR1
PR2
PR4
PR5

Fig. 3

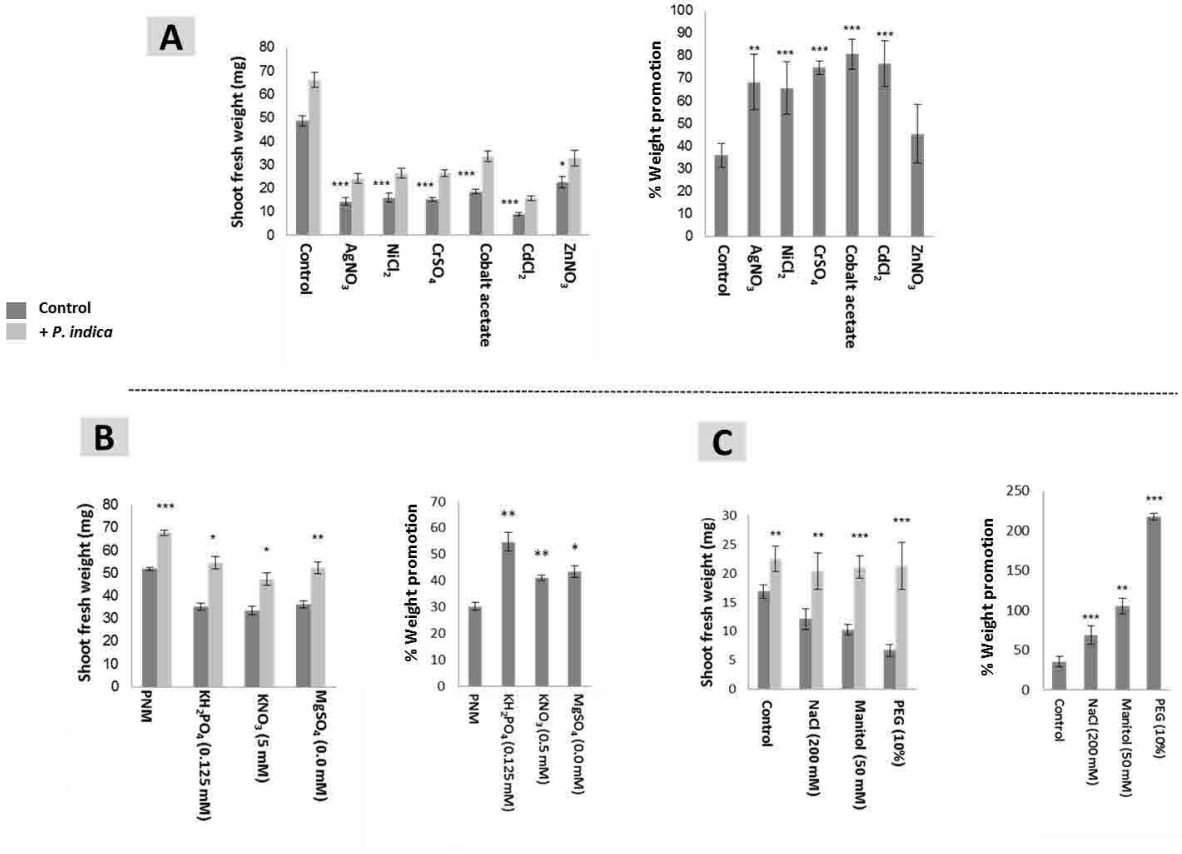
A
Shoot fresh weight (mg)
% Weight promotion
Control
AgNO3
NiCl2
CrSO4
Cobalt acetate
CdCl2
ZnNO3
Control
+ P. indica
B
Shoot fresh weight (mg)
% Weight promotion
PNM
KH2PO4 (0.125 mM)
KNO3 (5 mM)
MgSO4 (0.0 mM)
KNO3 (0.5 mM)
C
Shoot fresh weight (mg)
% Weight promotion
Control
NaCl (200 mM)
Manitol (50 mM)
PEG (10%)

Fig. 4

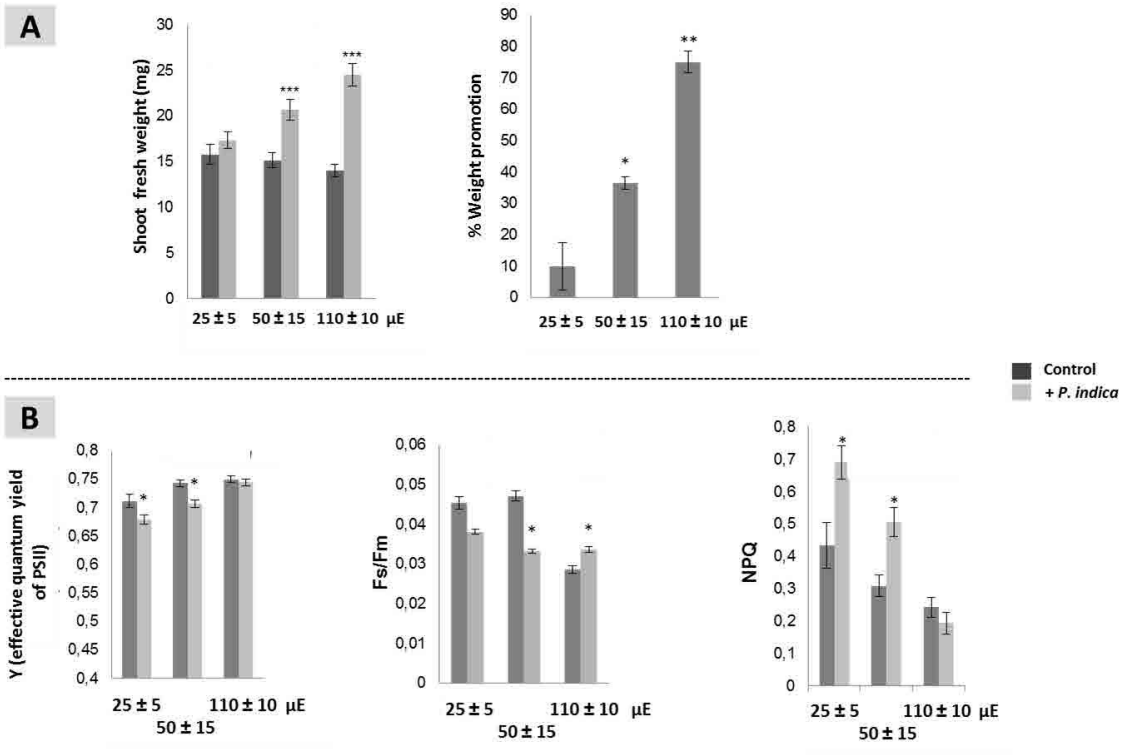
A
Shoot fresh weight (mg)
30
25
20
15
10
5
0
***
***
25 ± 5
50 ± 15
110 ± 10 µE
% Weight promotion
90
80
70
60
50
40
30
20
10
0
*
**
25 ± 5
50 ± 15
110 ± 10 µE
Control
+ P. indica
B
Y (effective quantum yield of PSII)
0,8
0,75
0,7
0,65
0,6
0,55
0,5
0,45
0,4
*
*
25 ± 5
50 ± 15
110 ± 10 µE
Fs/Fm
0,06
0,05
0,04
0,03
0,02
0,01
0
*
*
25 ± 5
50 ± 15
110 ± 10 µE
NPQ
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
*
*
25 ± 5
50 ± 15
110 ± 10 µE

Fig. 5

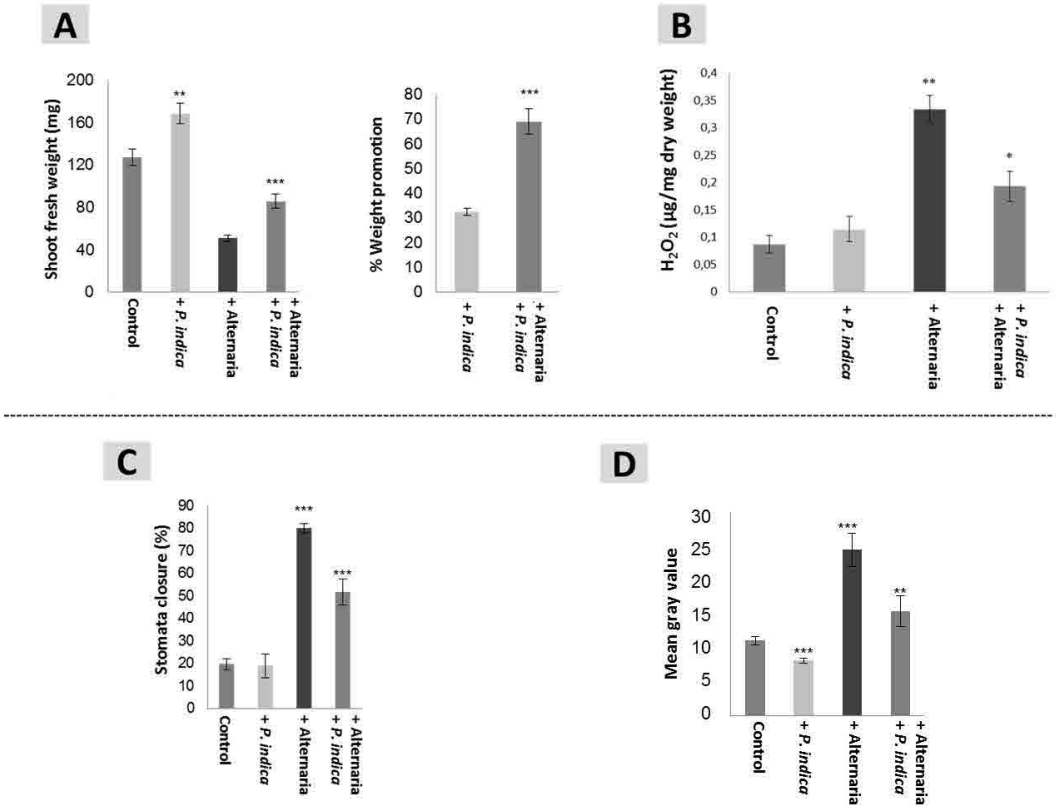
A
Shoot fresh weight (mg)
200
160
120
80
40
0
**
***
Control
+ P. indica
+ Alternaria
+ P. indica
+ Alternaria
% Weight promotion
80
70
60
50
40
30
20
10
0
***
+ P. indica
+ Alternaria
+ P. indica
B
H2O2 (µg/mg dry weight)
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
**
*
Control
+ P. indica
+ Alternaria
+ Alternaria
+ P. indica
C
Stomata closure (%)
90
80
70
60
50
40
30
20
10
0
***
***
Control
+ P. indica
+ Alternaria
+ P. indica
+ Alternaria
D
Mean gray value
30
25
20
15
10
5
0
***
***
**
Control
+ P. indica
+ Alternaria
+ P. indica
+ Alternaria

Fig. 6

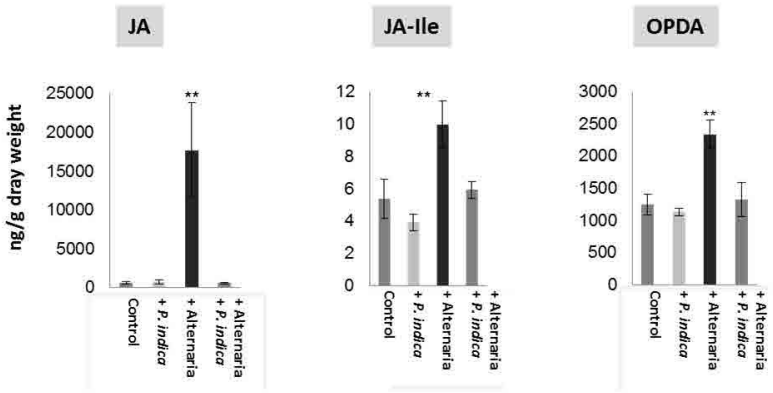
JA
JA-Ile
OPDA
ng/g dray weight
25000
20000
15000
10000
5000
0
12
10
8
6
4
2
0
3000
2500
2000
1500
1000
500
0
**
Control
+ P. indica
+ Alternaria
+ P. indica
+ Alternaria

Fig. 7

Electronic Supplementary Material

Role of stress in *P. indica*-Arabidopsis interaction

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Supplementary Materials

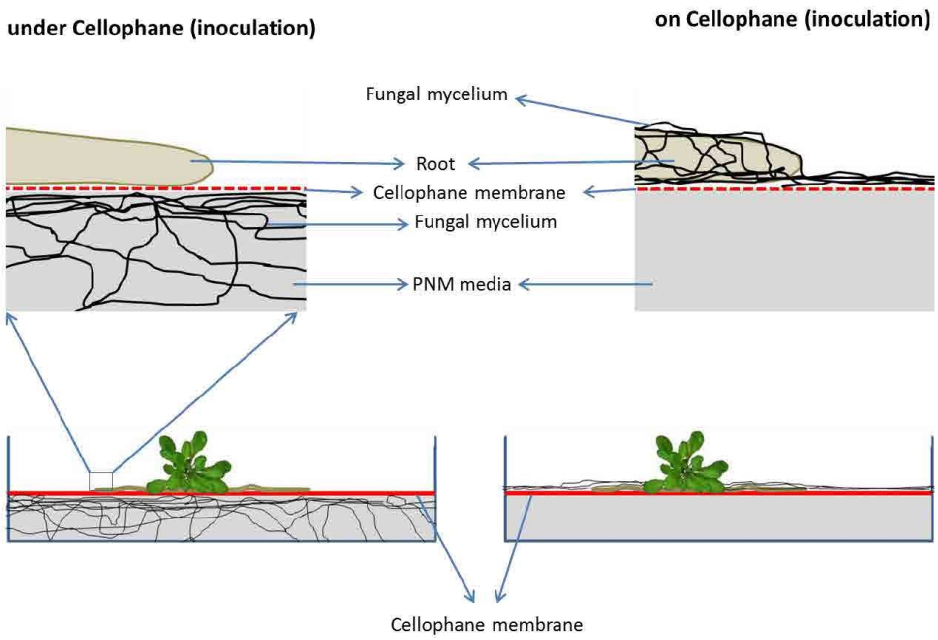

Figure S1. Scheme for co-cultivation of Arabidopsis seedlings with *P. indica* grown under the cellophane membrane (*left*) or on top of the cellophane membrane (*right*). In both cases seedlings are grown on the top of the cellophane membrane. The pore size of the cellophane membrane is too small for root hair and mycelia to penetrate. Thus communication between the two organisms can only occur *via* chemical mediators.

Table S1. List of primers used for Real-time PCR

Primer name	**5‘-3‘ forward primer**	**5‘-3‘ reverse primer**
At3g04120 (*GAPDH*)	GAG CTG ACT ACGTTG TTG AG	GGA GAC AAT GTC AAG GTC GG
At2g14610 (*PR1*)	GCTCAAGATAGCCCACAAGA	TGTATGAGTCTGCAGTTGCC
At3g57260 (*PR2*)	ACCACACAGCTGGACAAATCG	ATGAGCTCGATGTCAGAGCCA
At3g12500 (*PR-3*)	TCA TGG GGC TAC TGT TTC AAG	TATTGCTCTACC GCATAGACC
At3g04720 (*PR4*)	GACCTCGTGGTCAAGCTTCTT	TTGCTACATCCAAATCCAAGC
At1g75040 (*PR5*)	TCCTCGTGTTCATCACAAGC	CGT CAA AGTTGCAGCCTGTA
P. indica ITS	CAACACATGTGCACGTCGAT	CCAATGTGCATTCAGAACGA

4.4 Manuscript IV

Sun C, Shao Y, Vahabi K, Lu J, Bhattacharya S, Dong S, Yeh K-W, Sherameti I, Lou B, Baldwin I T, Oelmüller1 R (2014) The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection by down-regulation plant defense responses. *BMC Plant Biology* 14:268.

Sun et al. BMC Plant Biology 2014, 14:268
http://www.biomedcentral.com/1471-2229/14/268

BMC Plant Biology

RESEARCH ARTICLE Open Access

The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection by downregulation plant defense responses

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Abstract

Background: *Verticillium dahliae* (*Vd*) is a soil-borne vascular pathogen which causes severe wilt symptoms in a wide range of plants. The microsclerotia produced by the pathogen survive in soil for more than 15 years.

Results: Here we demonstrate that an exudate preparation induces cytoplasmic calcium elevation in Arabidopsis roots, and the disease development requires the ethylene-activated transcription factor EIN3. Furthermore, the beneficial endophytic fungus *Piriformospora indica* (*Pi*) significantly reduced *Vd*-mediated disease development in Arabidopsis. *Pi* inhibited the growth of *Vd* in a dual culture on PDA agar plates and pretreatment of Arabidopsis roots with *Pi* protected plants from *Vd* infection. The *Pi*-pretreated plants grew better after *Vd* infection and the production of *Vd* microsclerotia was dramatically reduced, all without activating stress hormones and defense genes in the host.

Conclusions: We conclude that *Pi* is an efficient biocontrol agent that protects Arabidopsis from *Vd* infection. Our data demonstrate that *Vd* growth is restricted in the presence of *Pi* and the additional signals from *Pi* must participate in the regulation of the immune response against *Vd*.

Keywords: Calcium, Defense, Ethylene, Jasmonic acid, *Piriformospora indica*, Salicylic acid, *Verticillium dahliae*

Background

Verticillium species are wide-spread soil-borne fungi which cause vascular diseases in many plant species and are responsible for devastating diseases for plants that can thwart agricultural production. The vascular wilt fungus *Verticillium dahliae* (*Vd*), for instance, infects more than 200 plant species, among them agriculturally and horticulturally important crops and ornamental plants [1-3]. It is estimated that *Vd* infections are responsible for several billions of dollars of annual crop losses worldwide. *Vd* has a broad host range and infects plants from temperate to subtropical climates [1]. Because of their complex life style of the Verticillium species, their control by classical pesticides or fungicides is difficult; therefore, the isolation of Verticillium-resistant cultivars is an important task for the breeders (cf. [4,5]).

Genetic resistance against Verticillium wilt diseases has been reported for several plant species [1,2]. The *Ve* gene provides resistance against race 1 isolates of *Vd* in tomato [6,7] and the tomato gene is also functional after expression in Arabidopsis [8]. Many studies have used Arabidopsis for the isolation of *Vd*-resistant germplasm [9,10] or the identification of novel resistance traits following mutagenesis [2,10-14]. Furthermore, quite recently, a large number of proteins and metabolites from different organisms as well as phytohormones have been described to be involved in establishing partial resistance against Verticillium wilt [15-22].

Like other Verticillium species, *Vd* can overwinter as mycelium in host plants or soil. The fungus can also form

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Sun et al. BMC Plant Biology 2014, 14:268
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seed-like structures called microsclerotia, long-lived survival structures of clusters of melanized cells with thick walls, which survive in the soil without a host plant or in association with plant material for up to 20 years [23,24]. The microsclerotia germinate in response to stimuli from root exudates [25]. The hyphae penetrate and grow inter- and intracellularly through the root cortex toward the central cylinder of the root [26,27]. They enter the xylem cells of the root, from where they colonize the xylem of the hypocotyl and leaves. Ultimately, the water transport is disrupted which results in the wilt phenotype [1-3]. Verticillium species are considered as hemibiotroph: a biotrophic phase within root xylem without a visible disease phenotype is followed by a necrotrophic phase in the aerial parts of the plant.

The spread of the pathogen occurs primarily by root infections from the soil. Therefore rhizosphere bacterial strains such as *Pseudomonas putida* B E2, *Pseudomonas chlororaphis* K15 or *Serratia plymuthica* R12 [28] or bacterial isolates [29] have been shown to function as efficient biocontrol agents against *Vd* spread. The microbial bioagents induce antibiosis, parasitism, competition and secretion of enzymes such as glucose oxidase, chitinase and glucanase which results in the induction of disease resistance in the hosts [12,30].

To our knowledge, there is no report on endophytic fungi which can be used as biocontrol agent against *Vd* in Arabidopsis. *Piriformospora indica* (*Pi*), a cultivable basidiomycete of Sebacinales, colonizes the roots of many plant species including Arabidopsis [31,32]. Like other members of Sebacinales, *Pi* is found worldwide in association with roots [33] and stimulates growth, biomass and seed production of the hosts [31,34-36]. The fungus promotes nitrate and phosphate uptake and metabolism [35,37]. *Pi* also confers resistance against abiotic [38,39] and biotic stress [40].

Here, we demonstrate that *Pi* is an efficient biocontrol agent that protects Arabidopsis from *Vd* infection *in vitro* and *in vivo* by inhibiting growth of *Vd* in roots. Furthermore, we give evidence that a *Vd*-exudate compound induces cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevation and the *Vd*-disease development is dependent on the ethylene-activated transcription factor EIN3.

Results

Pi inhibits growth of *Vd* on PDA agar plates

Pi and *Vd* were co-cultivated as described in Methods on a PDA agar plate for 3 weeks. Figure 1(A and B) demonstrates that *Pi* strongly inhibits growth of *Vd* hyphae. The *Vd* colony in the dual culture is significantly smaller than the *Vd* colony growing without *Pi*. Furthermore, the number of microsclerotia produced by *Vd* in the dual culture is less than the number of microsclerotia produced by *Vd* growing alone. No obvious inhibition zone can be detected. In contrast, growth of *Pi* is barely affected by the presence of *Vd*. This prompted us to test the role of *Pi* in protecting Arabidopsis plants against *Vd* infection.

Arabidopsis seedlings pretreated with *Pi* are protected against *Vd* infection

To investigate whether *Pi* can protect Arabidopsis for *Vd* infection, we exposed the seedlings first to *Pi* prior to *Vd* infection. Seedlings not exposed to any of the two fungi or to one of the two fungi alone served as controls (cf. Methods). The performance of the seedlings was measured after 10, 14 and 21 days, by visible inspection and measuring the fresh weights. After 10 days of co-cultivation, seedlings treated with *Vd* or *Pi* alone showed ~30% increase in the biomass compared to the untreated control seedlings. A comparable increase in the biomass was observed when the seedlings were first exposed to *Pi* and then to *Vd* or *vice versa* (Figure 2A). This slight

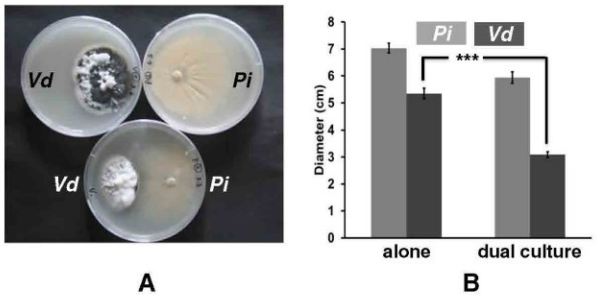

Figure 1 *Pi* inhibits growth of *Vd* on agar plates. (A) Typical plates from 3 independent experiments are shown. **(B)** Quantification of the colony. The diameter of the *Pi* and *Vd* mycelia on the agar plate is given in cm. Bars represent SDs. Asterisks indicate significant differences, as determined by ANOVA (*** $P \leq 0.001$).

Sun et al. BMC Plant Biology 2014, 14:268
http://www.biomedcentral.com/1471-2229/14/268

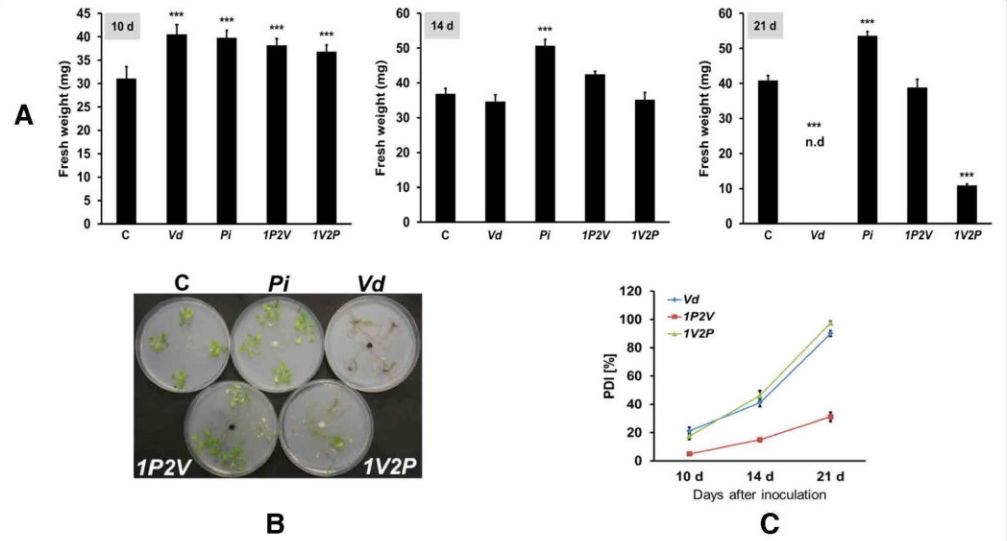

Figure 2 *Pi* protects Arabidopsis seedlings from *Vd* infection. (A) Fresh weights of seedlings after 10, 14 and 21 days of co-cultivation or mock-treatments on Petri dishes. The seedlings were exposed to either *Pi* or *Vd* alone or in combination as described in the Methods and Additional file 1: Figure S1. C: seedlings treated without fungi; *Vd*: seedlings treated with *Vd*; *Pi*: seedlings treated with *Pi*; *1P2V*: seedlings first treated with *Pi* for 4 days followed by *Vd*; *1V2P*: *vice-versa* as *1P2V*. n.d: no detectable (seedlings were dead, no fresh weight could be determined). The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences, as determined by ANOVA (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). **(B)** The phenotype of typical seedlings on 21st day. **(C)** PDI for seedlings exposed to *Vd*. For treatments, cf. Methods and Additional file 1: Figure S1. The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs.

increase in the biomass suggests that both fungi initially form a beneficial interaction with the seedlings, and is consistent with the idea that this phase represents a biotrophic interaction of *Vd* with Arabidopsis roots. On the 14th day, seedlings infected by *Vd* alone or first with *Vd* followed by *Pi* (*1V2P*) showed obviously the disease symptoms. The leaves of these seedlings became paler and the roots browner compared to the seedlings exposed to *Pi* or *1P2V* treatments, although no significant differences in the biomass were observed for the different fungal treatments, except for *Pi* treatment (Figure 2A). In contrast, on the 21st day, seedlings exposed to *Vd* alone or exposed to *Vd* prior to exposure to *Pi* (*1V2P*) were severely damaged. Their fresh weights were reduced or no longer measurable. *Pi* treatment alone resulted in a ~30% increase in the fresh weight (Figure 2A). Interestingly, seedlings which were pretreated with *Pi* and then exposed to *Vd* (*1P2V*) had the same fresh weights as untreated control seedlings, although the visible inspection showed some photobleaching (Figure 2B). This clearly demonstrates that *Pi* protects Arabidopsis seedlings against *Vd*-induced wilt. Therefore, this experimental set-up was used to study the protective function of *Pi* in greater details.

The results were confirmed by calculating the Percentage Disease Index (PDI) for those seedlings treated with *Vd*. After 10 days of co-cultivation, the PDI for *Vd* and *1V2P* seedlings was ~20%, and after 14 days 40-50%. After 21 days, the PDI was almost 100%. In contrast, seedlings pretreated with *Pi* prior to exposure to *Vd* (*1P2V*) showed a slow increase in the PDI, which reached ~30% after 21 days (Figure 2C).

Furthermore, the amount of total chlorophyll (Chl) is a sensitive marker for the fitness of a plant. On the 4th day, the shoots of *Vd*- and *Pi*- treated plants contained slightly higher Chl levels than control seedlings (Figure 3). On the 10th day, the Chl content of *Vd*-treated seedlings is comparable to that of control seedlings not exposed to the pathogen. Furthermore, while *1P2V* seedlings had the same amount of Chl as *Pi* seedlings, the Chl content in *1V2P* seedlings was significantly reduced (Figure 3). Comparable results were obtained for the 14th day, except that the Chl content for *1P2V* seedlings was reduced compared to *Pi* seedlings (Figure 3). On the 21st day, *Pi* seedlings had the highest Chl content, *1P2V* seedlings had the same amount of Chl as control seedlings not exposed to a fungus, while

Sun et al. BMC Plant Biology 2014, 14:268
http://www.biomedcentral.com/1471-2229/14/268

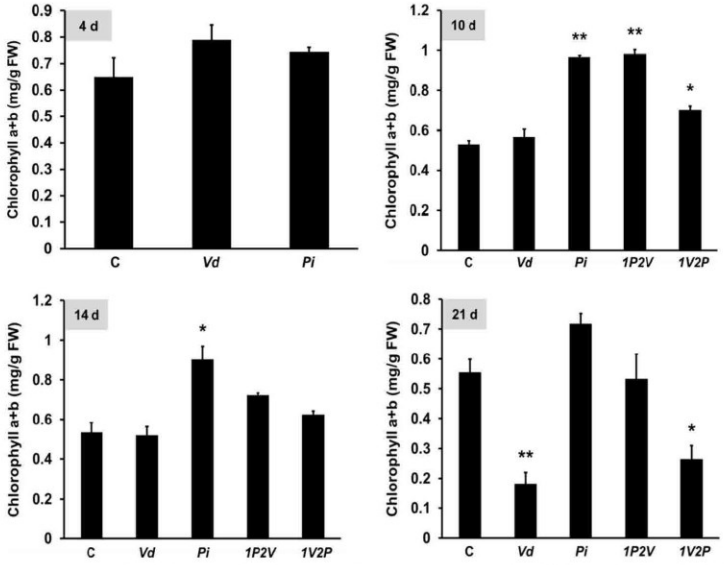

Figure 3 Total chlorophyll content (mg/g fresh weight) in shoots. The data were obtained 4, 10, 14 and 21 days after the fungal treatments (cf. Methods, Additional file 1: Figure S1 and legend to Figure 2A). The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences to the untreated control, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

the Chl levels in the *Vd* and *1V2P* plants were strongly decreased (Figure 3). This confirms the protective function of *Pi* against *Vd* infection in Arabidopsis leaves.

Pathogenesis and application of pathogen-associated molecular patterns induce stomata closure [41]. In control plants not exposed to any fungus, between 5 and 12% of the stomata were closed. Three days after exposure of the roots to *Vd*, ~25% of the stomata were closed (Figure 4A), and this increased to ~30% until the 7th day. The *1V2P* treatment showed ~25% stomata closure at the 7th day, and this value is comparable to that for seedlings treated with *Vd* alone. In contrast, exposure of the roots to *Pi* or first to *Pi* followed by *Vd* did not result in stomata closure and these values are comparable to those of the untreated controls (Figure 4B). This indicates that *Pi* prevents *Vd*-induced stomata closure. These results demonstrate that stomatal closure correlates nicely with the amount of total chlorophyll.

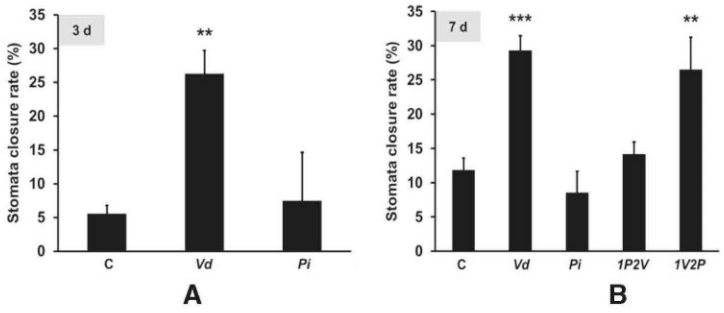

Figure 4 Stomata closure rate in leaves after 3 (A) and 7 (B) days. The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences to the untreated control, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Pi represses *Vd*-induced genes in shoots

Vd induces defense gene expression in shoots. After 1 d, the mRNA levels for *PR1* and *PR2* representing SA-inducible genes and *PDF1.2* for the JA/ET pathway, *ERF1* and *VSP2* for ET pathway were upregulated in the leaves of *Vd*-exposed seedlings. Except for *PR2*, none of the other genes responded to *Pi* exposure (Figure 5). After 14 d, *Vd*-exposed seedlings showed an even stronger up-regulation of the defense genes in the leaves (Figure 5). Pretreatment of the seedlings with *Pi* prior to *Vd* infection resulted in the repression of defense gene expression compared to seedlings which were not pretreated with *Pi*. This provides additional evidence for the protective function of *Pi* against *Vd* infection. Furthermore, plant glutamate receptor-like (*GLR*) genes, *GLR2.4*, *GLR2.5* and *GLR3.3* code for putative Ca^{2+} transporters and are involved in defense responses [42-44]. We observed that *GLR2.4* (but not *GLR2.5* and *GLR3.3*) was upregulated in the leaves of *Vd*-exposed seedlings and repressed in the leaves of seedlings which were pretreated with *Pi* prior to *Vd* exposure (Figure 5 and Additional file 1: Figure S2). *RabGAP22* is required for defense to *V. longisporum* and contributes to stomata immunity [22]. For *Vd*, *RabGAP11* is upregulated after exposure to *Vd* and significantly repressed in seedlings which were pretreated with *Pi* (Figure 5).

Pi strongly represses *Vd*-induced phytohormone accumulation in shoots

The phytohormones JA, JA-Ile, OPDA, SA, ABA and ET are crucial for the activation of defense responses. Figure 6 demonstrates that these phytohormones accumulated after *Vd* infection in the shoots of Arabidopsis seedlings. The phytohormone levels were also high in the *1V2P* samples, while in all other cases [Control (C), *Pi*, *1P2V*], they showed significantly lower levels. Thus, *Vd*-induced phytohormone accumulation is repressed if the roots are colonized by *Pi* prior to their exposure to *Vd*. Interestingly, application of *Pi* to roots which were already exposed to *Vd* did not repress the accumulation of the phytohormones in the shoots.

Pi inhibits *Vd* propagation and microsclerotia formation

Quantification of the amount of *Vd* DNA demonstrated that *Vd* and *1V2P* seedlings contain twice as much pathogen DNA than *1P2V* seedlings in both roots (Figure 7A and D) and shoots (Figure 7B and E). Interestingly, the amount of *Pi* DNA in the roots is identical in all *Pi*-treated samples and not affected by a pretreatment with *Vd* (Figure 7C and F). Furthermore, microscopic analysis demonstrated that the number of microsclerotia was strongly reduced in root tissue pretreated with *Pi* (Figure 8). This demonstrates that *Pi* inhibits *Vd* propagation and microsclerotia formation in the roots, while *Vd* does not affect the propagation of *Pi* in Arabidopsis roots.

Long-term experiments confirmed the results obtained for seedlings

In order to study long term interaction, the seedlings were grown according to the 5 regimes on Petri dishes for 10 days before transferred to sterile vermiculite for additional 14 days. All (C) seedlings and those exposed to *Pi* (*Pi*) were alive. Exposure of *Pi*-pretreated plants to *Vd* resulted in ~20% loss of the plants. However 80% of the plants, which were either exposed to *Vd* alone or first to *Vd* followed by *Pi*, died (Figure 9A). Furthermore, we measured the fresh weights of the seedlings which survived the treatments. Plants exposed to *Pi* alone showed a ~30% increase in the fresh weight. The fresh weights of *1P2V* plants were comparable to those not exposed to any fungus. *Vd*- and *1V2P*-treated seedlings showed significantly decreased fresh weights compared to all other treatments (Figure 9B). Finally, the *Vd* DNA amount in both shoots and roots was lower in *1P2V*-treated plants compared to those treated with *Vd* alone or first with *Vd* followed by *Pi* (*1V2P*) (Figure 9C). Comparable to the results obtained with seedlings in Petri dishes (Figure 7), the *Pi* DNA content was the same in all *Pi*-treated roots (Figure 9C). This confirms that *Pi* inhibits *Vd* growth, but not *vice versa*.

EIN3 is required for full susceptibility of Arabidopsis to *Vd*

The strong upregulation of the phytohormone levels in the leaves of seedlings grown in the presence of *Vd* was further investigated for ET. Pantelides et al. [11] have shown that ET perception *via* ETR1 is required for *Vd* infection in Arabidopsis. We observed a strong requirement of EIN3 for *Vd*-induced disease development in Arabidopsis leaves. *ein3* seedlings which were exposed to *Vd* alone or were first treated with *Vd* before application of *Pi* perform better than wild-type seedlings (Figure 10A, B and Additional file 1: Figure S3). Interestingly, the ET level in *ein3* seedlings is much higher than in wild-type seedlings, even in the absence of *Vd*. Exposure of the seedlings to *Vd* stimulate ET accumulation even further (Figure 10C and Additional file 1: Figure S4). This suggests that *ein3* seedlings try to compensate the lack of EIN3-induced genes by further stimulating ET biosynthesis, in particular after *Vd* infection. Taken together, these data demonstrate that EIN3-induced genes are required for pathogenicity of *Vd*.

Vd induces $[Ca^{2+}]_{cyt}$ elevation in WT roots, but not in roots of a Ca^{2+} response mutant

Pathogen-associated molecular pattern-triggered immunity is often initiated by $[Ca^{2+}]_{cyt}$ elevation, which can be induced by exudated compounds from pathogenic fungi [cf. [45] and ref. therein]. Since the putative plasma membrane-localized Ca^{2+}-transporter gene *GLR2.4* was upregulated by *Vd*, we tested whether exudated compounds

Sun et al. BMC Plant Biology 2014, 14:268
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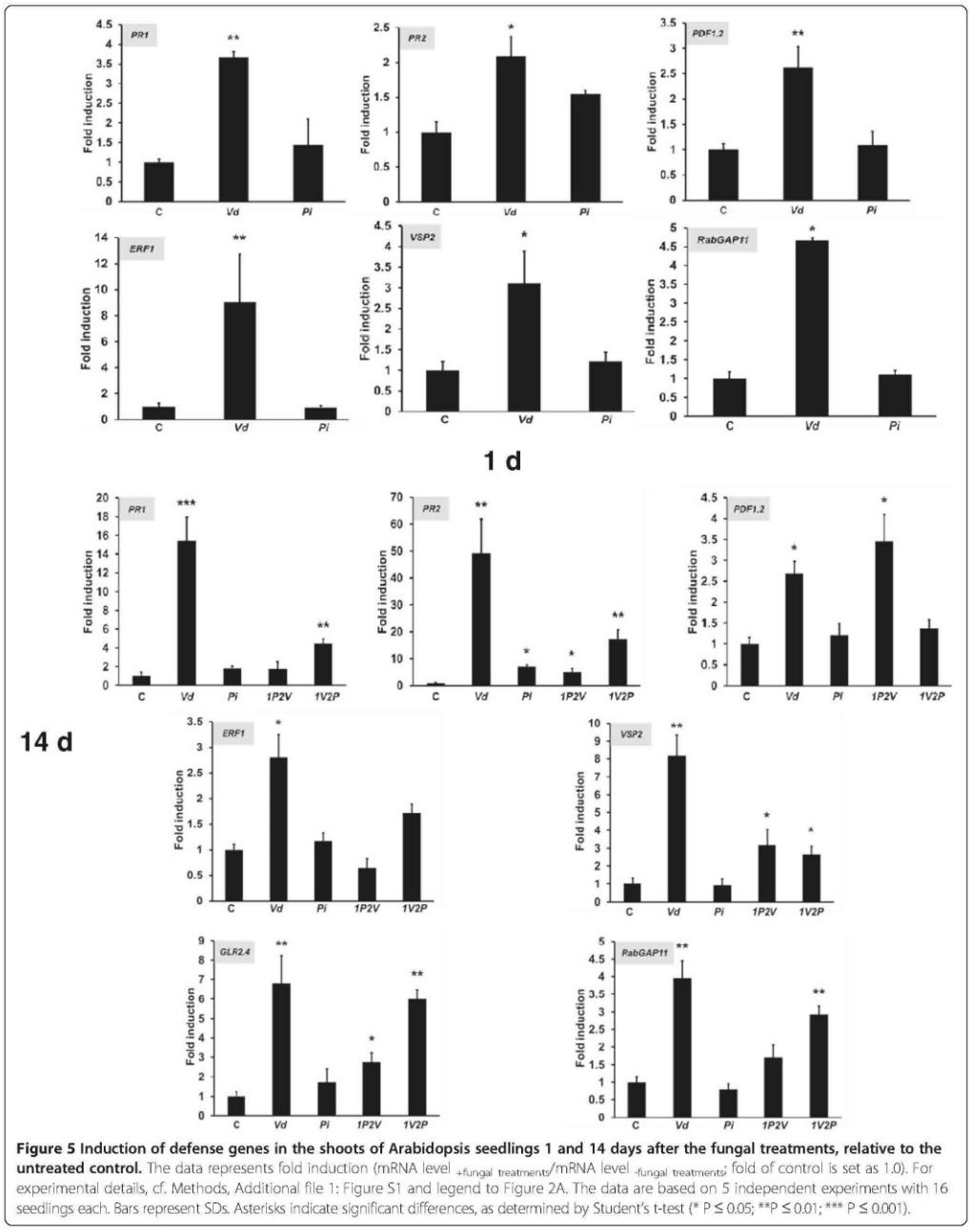

Figure 5 Induction of defense genes in the shoots of Arabidopsis seedlings 1 and 14 days after the fungal treatments, relative to the untreated control. The data represents fold induction (mRNA level $_{+\text{fungal treatments}}$/mRNA level $_{-\text{fungal treatments}}$; fold of control is set as 1.0). For experimental details, cf. Methods, Additional file 1: Figure S1 and legend to Figure 2A. The data are based on 5 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Sun et al. BMC Plant Biology 2014, 14:268
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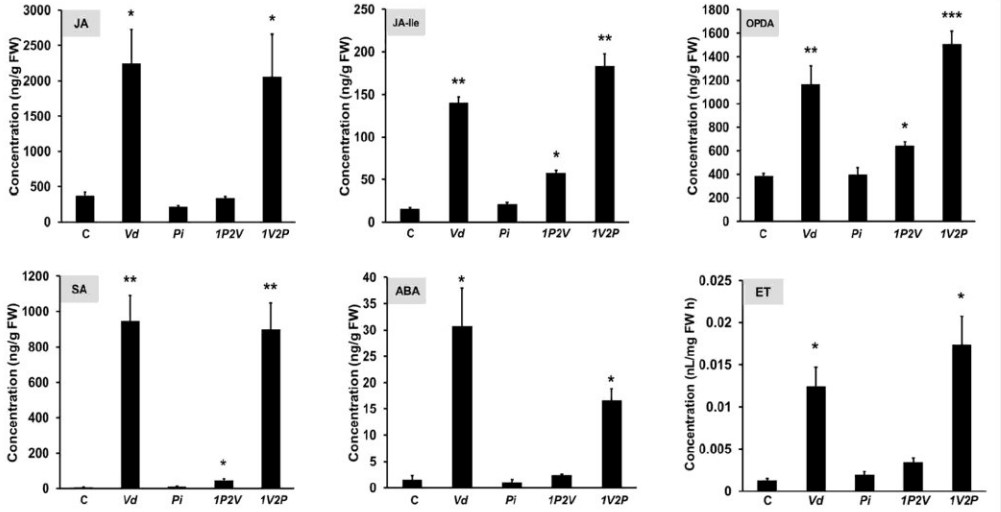

Figure 6 Phytohormone levels in the shoots 21 days after the different fungal treatments. For experimental details, cf. Methods, Additional file 1: Figure S1, and legend to Figure 2A. The data are based on 3 independent experiments with 12 seedlings each. Bars represent SDs. Asterisks indicate significant differences, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

from *Vd* can induce $[Ca^{2+}]_{cyt}$ elevation in roots. An exudate preparation from the mycelium was applied to the roots of transgenic pMAQ2 Arabidopsis lines expressing the Ca^{2+}-sensor apoaequorin. Under resting conditions, 21 d-old pMAQ2 lines gave $[Ca^{2+}]_{cyt}$ values of 70 ± 0.6 nM ($n = 16$). A rapid and transient increase in the $[Ca^{2+}]_{cyt}$ concentration is observed 40 sec after the application of *Vd* preparation (Figure 11A). Discharge at the end of the experiment demonstrates that less than 5% of the reconstituted aequorin was consumed after the stimuli, which ensures that the amount of aequorin in the sample is not limiting for the Ca^{2+} signal (data not shown). The $[Ca^{2+}]_{cyt}$ reached a peak of ~ 400 nM after 90 to 120 sec (Figure 11A). Subsequently the Ca^{2+} levels steadily decreased. No $[Ca^{2+}]_{cyt}$ elevation is observed with the PBS buffer treatment (Figure 11A). The magnitude of the $[Ca^{2+}]_{cyt}$ response is dose-dependent (data not shown). Furthermore, an Arabidopsis *cytoplasmic calcium elevation mutant1* (*cycam1*) which does not show $[Ca^{2+}]_{cyt}$ elevation in response to exudate preparation from various pathogenic fungi [45] also failed to induce $[Ca^{2+}]_{cyt}$ elevation in response to the *Vd* preparation (Figure 11B). This indicates that *cycam1* is impaired in the response to exudate preparations from various pathogens. Furthermore, we crossed the apoaeqorin gene into the *glr2.4*, *glr2.5* and *glr3.3* knock-out background. Figure 11B demonstrates that the *Vd* exudate preparation induced $[Ca^{2+}]_{cyt}$ elevation in the knock-out backgrounds, indicating that these putative plasma membrane-localized transporters do not participate in the Ca^{2+} uptake from the extracellular space, although the gene *GLR2.4* was upregulated in *Vd*-infected seedlings (Figure 5).

To investigate whether $[Ca^{2+}]_{cyt}$ elevation is required for disease development, *cycam1* was infected with *Vd* and the development of the mutant seedlings was compared to that of the WT seedlings. No obvious difference of the disease symptoms in the aerial parts could be detected, which suggests that $[Ca^{2+}]_{cyt}$ elevation is not essential for *Vd* propagation (Additional file 1: Figure S6).

Discussion

Our data demonstrate that *Pi* is a very efficient biocontrol agent for *Vd* wilt in Arabidopsis. *Pi* restricts *Vd* growth both on agar plates (Figure 1) and in Arabidopsis roots, in particular when they were first colonized by *Pi* prior to infection with *Vd* (Figure 7). Molecular and biochemical analyses demonstrate that the reduced growth rate of *Vd* in *Pi*-pretreated Arabidopsis roots retards defense gene expression (Figure 5), the accumulation of defense-related phytohormones (Figure 6) and stomata closure (Figure 4). The performance of the seedlings is significantly better (Figure 2) and this also continues after shifting the seedlings to vermiculite for a longer period of time (Figure 9). *Pi* not only inhibits growth of *Vd* mycelia in Arabidopsis roots, but also prevents the spread of the pathogen to the aerial parts of the plant (Figure 7). Furthermore, microsclerotia formation is strongly reduced (Figure 8). Previously, several soil-borne bacteria have been identified as biocontrol agents for Verticillium wilt

Sun et al. BMC Plant Biology 2014, 14:268
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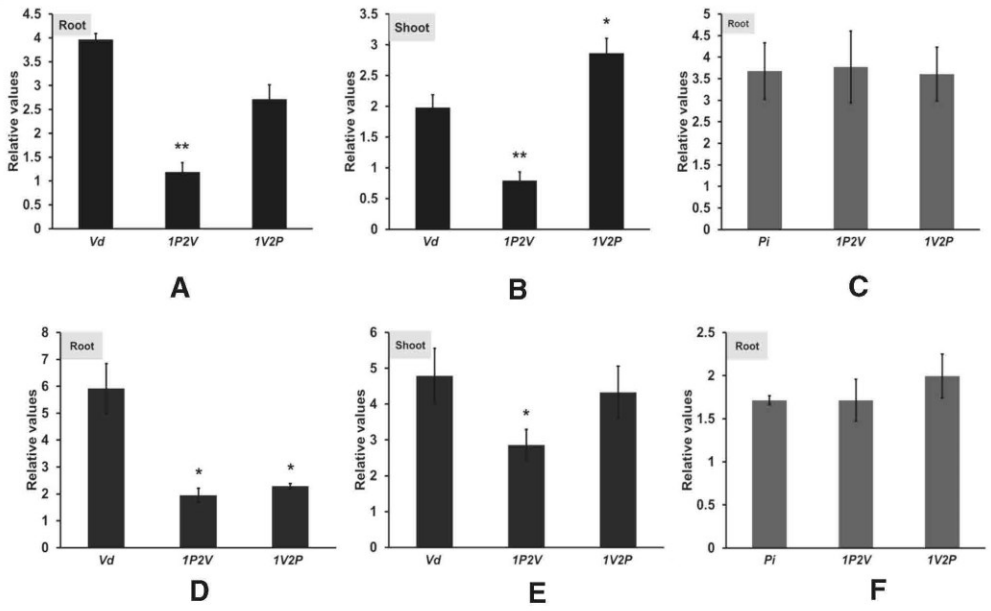

Figure 7 The amount of fungal DNA in the roots and shoots of Arabidopsis seedlings exposed to the 5 treatments (cf. legend to Figure 2A). For experimental details, cf. Methods and Additional file 1: Figure S1. The measurements were performed for the 14th **(A, B, C)** and 21st **(D, E, F)** day. The data are based on 3 independent experiments with 12 seedlings each. Bars represent SDs. Asterisks indicate significant differences compared to *Vd* **(A, B, D, E)** or to *Pi* **(C and F)**, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

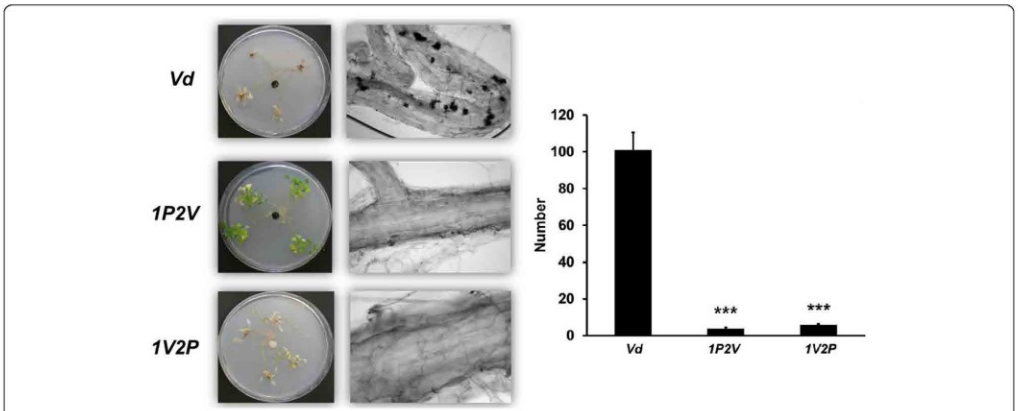

Figure 8 *Pi* inhibits the formation of *Vd* micosclerotia in roots, irrespective of whether the roots were first exposed to *Pi* (*1P2V*) or first to *Vd* (*1V2P*). The analysis was performed 21 days after infection. Left: microscopy of root sections with microslerotia (black spots). Right: Quantification of the number of microsclerotia. The data are based on 3 independent experiments with 12 seedlings each. Bars represent SDs. Asterisks indicate significant differences to *Vd*, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Sun et al. BMC Plant Biology 2014, 14:268
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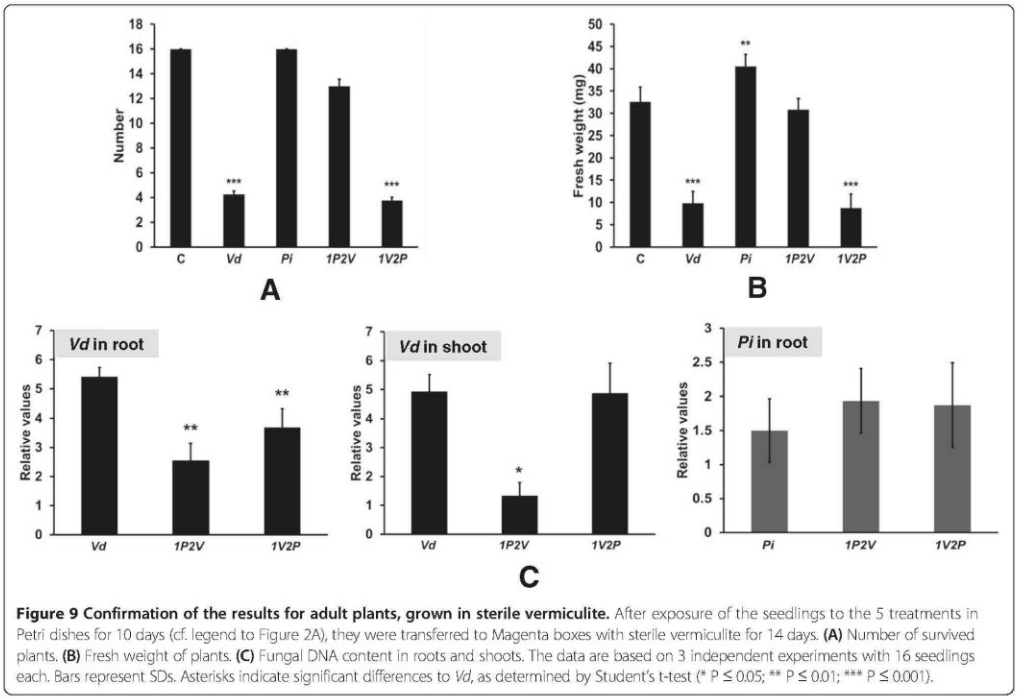

Figure 9 Confirmation of the results for adult plants, grown in sterile vermiculite. After exposure of the seedlings to the 5 treatments in Petri dishes for 10 days (cf. legend to Figure 2A), they were transferred to Magenta boxes with sterile vermiculite for 14 days. **(A)** Number of survived plants. **(B)** Fresh weight of plants. **(C)** Fungal DNA content in roots and shoots. The data are based on 3 independent experiments with 16 seedlings each. Bars represent SDs. Asterisks indicate significant differences to *Vd*, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

[29,46-48]. *Vd* can induce antimicrobial metabolites such as rutin in potato [49] or pathogenesis-related proteins in Arabidopsis [12] which participates in pathogen resistance. Prieto et al. [50] demonstrated that root hair colonization plays an important role in *Pseudomonas* spp.-mediated biocontrol activity against Verticillium wilt in olive roots. Furthermore, the *Bacillus subtilis* strain NCD-2 functions as a biocontrol agent against cotton Verticillium wilt, and the cotton PhoR/PhoP, two component regulatory systems, were involved in the biocontrol capability of the bacterium [51]. Also quorum sensing is involved in the biocontrol activity of *Serratia plymuthica* against *Vd* [52]. Moderate drought influences the effect of arbuscular mycorrhizal fungi as biocontrol agents against Verticillium-induced wilt in pepper [53]. It appears that quite different mechanisms control the fungal spread, probably because of the complicated lifestyle of the pathogen which allows microbial interference at different levels and in different plant tissues.

An increasing number of genes were recently identified to be involved in establishing partial resistance to Verticillium wilts (cf. Background). Pathogen attack including root colonization by *Vd* is associated with stomata closure as one of the first line of plant defense (Figure 4). *RabGAP22* is required for defense against *V. longisporum* and contributes to stomatal immunity [20]. *RabGAP11* gene is upregulated by *Vd* and repressed by *Pi* (Figure 5). Finally, defensins play a role in the plant defense against *Vd* [19].

Control of microsclerotia formation is crucial for preventing Verticillium spread in nature and agriculture. Our data demonstrate that *Pi* is quite efficient in restricting microsclerotia formation in Arabidopsis roots (Figure 8), presumable because the pathogen cannot grow fast enough in the presence of *Pi*. Microsclerotia formation is also suppressed by Verticillium itself, i.e. by the fungal transcription activator of adhesion Vta2, and fungi impaired in Vta2 are unable to colonize plants and induces disease symptoms [21]. Taken together, *Pi* restricts *Vd* growth as well as hyphal and microslerotia propagation, which - in turn - causes that the plant defense processes get activated at a lower level compared to *Vd* treatments which might depend on *Pi*-plant-*Vd* interaction-pattern and the attack strategy of *Vd*. This is not only important for better performance of individual plants, but has also severe long-term consequences for the control of the *Vd* spread *via* microsclerotia in ecosystems and agricultural areas.

GRL homologs are associated with Ca^{2+} influx through the plasma membrane. Figure 5 demonstrates that the

Sun et al. BMC Plant Biology 2014, 14:268
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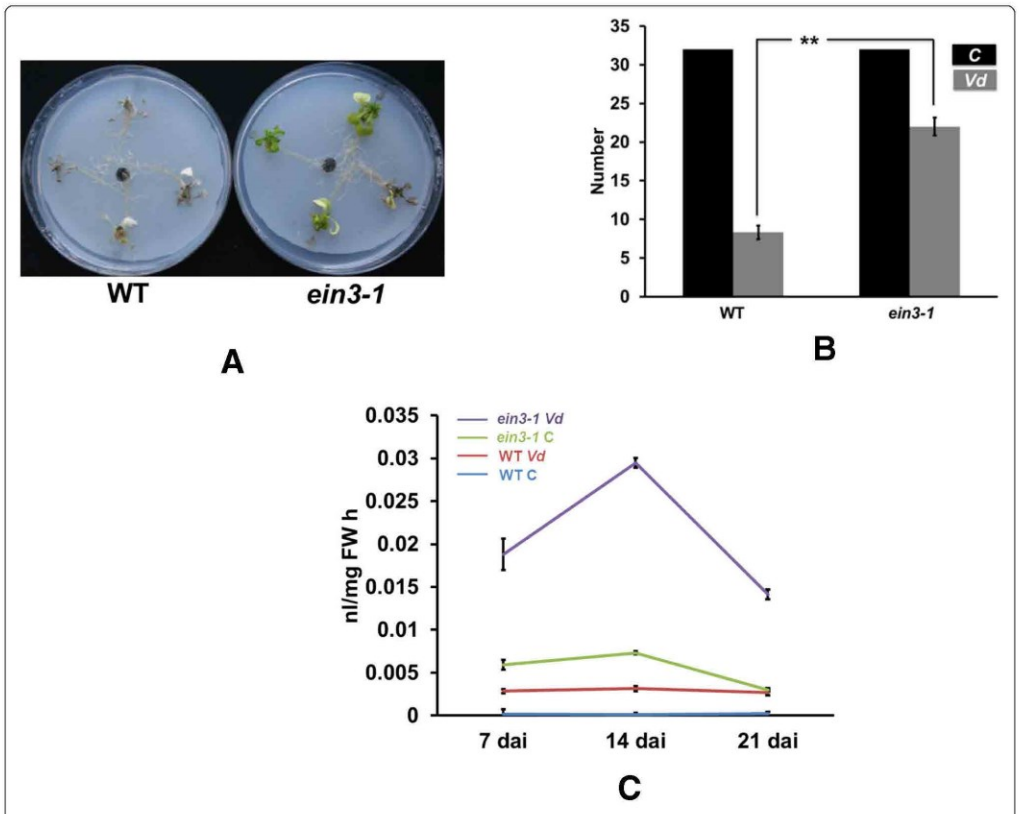

Figure 10 EIN3 is required for full susceptibility of Arabidopsis to *Vd*. **(A)** The representative picture (3 independent experiments with 32 plants each) was taken after 21 days inoculation with *Vd*. **(B)** Number of survived seedlings. **(C)** Ethylene levels in WT and *ein3* seedlings after exposure to *Vd*. Bars represent SDs. Asterisks indicate significant differences, as determined by Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

mRNA level for *GLR2.4* is upregulated in the leaves of *Vd*-infected Arabidopsis seedlings and these responses are restricted by a pretreatment of the seedlings with *Pi*. GLR3.3 is involved in plant defense and resistance to *Hyaloperonospora arabidopsidis* [44]. The protein also mediates glutathione-triggered $[Ca^{2+}]_{cyt}$ transients, transcriptional changes, and innate immunity responses in Arabidopsis [54]. *GLR2.5* is upregulated in Arabidopsis cell cultures upon wounding [43] and *GLR2.4* is induced by nematodes in Arabidopsis roots [42]. GLR2.4, also called AUGMIN subunit 8, is a microtubule plus-end binding protein that promotes microtubule reorientation in hypocotyls [55,56]. Microtubules and microtubule orientation are important for plant defense and immunity [56,57] and also involved in *Vd*-Arabidopsis interaction. Hu et al. [18] demonstrated that histone H2B monoubiquitination is involved in regulating the dynamics of microtubules during the defense response to *Vd* toxins in Arabidopsis. Yuan et al. [58] showed that *Vd* toxins disrupted microfilaments and microtubules in Arabidopsis suspension-cultured cells. Figure 11A shows that exudate compounds from *Vd* induces $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis roots. In order to test whether the $[Ca^{2+}]_{cyt}$ elevation is mediated by one of the three GLRs, we generated transgenic *glr3.3*, *glr2.5* and *glr2.4* knock-out lines in the apoaequorin background and found that the $[Ca^{2+}]_{cyt}$ response is not controlled by the three GLRs (Figure 11B), although the mRNA level of *GLR2.4* is upregulated upon *Vd* infection (Figure 5). This suggests that GLRs have different functions in the *Vd*-Arabidopsis interaction. However, an ethylmethansulfonate-induced Arabidopsis mutant named *cycam1* which is unable to

Sun et al. BMC Plant Biology 2014, 14:268
http://www.biomedcentral.com/1471-2229/14/268

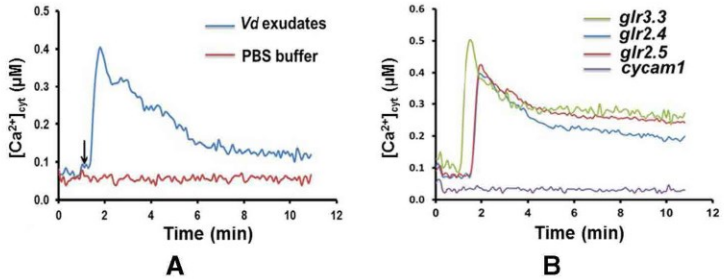

Figure 11 ***Vd*** **exudate preparation induces** $[Ca^{2+}]_{cyt}$ **elevation in** ***A. thaliana*** **seedlings expressing cytosolic aequorin. (A)** Roots of 21-day old pMAQ2 in Col-0 seedlings were dissected and incubated overnight in 7.5 μM coelenterezine. The roots were challenged with 50 μl of the *Vd* preparations. $[Ca^{2+}]_{cyt}$ level was calculated from the relative light unit (RLU) at 5 s integration time for 10 min. The arrow indicates the time (60 s) of addition of the stimuli/PBS buffer. For all experiments, 10 mM phosphate buffer (PBS, pH 7.0) was used as control and gave background readings. All curves and values represent average of five independent experiments with eight replications in each experiment. **(B)** *Vd* exudate preparation does not induce $[Ca^{2+}]_{cyt}$ elevation in the *cycam1* mutant, but induces $[Ca^{2+}]_{cyt}$ elevation in pMAQ2 lines in the *glr2.4*, *glr2.5* and *glr3.3* background.

induce $[Ca^{2+}]_{cyt}$ elevation in response to exudate preparations from *Alternaria brassicae*, *Rhizoctonia solani*, *Phytophthora parasitica* var. *nicotianae* and *Agrobacterium tumefaciens* [45] did not respond to the *Vd* exudate preparation (Figure 11B). This demonstrates that at least one of the *Vd*-induced signaling events leading the opening of Ca^{2+} channels or the channels themselves are identical to those responding to exudate preparations from other pathogens [45]. However, the reduced Ca^{2+} response in the *cycam1* mutant does not affect the disease development. It remains to be determined which is the active compound inducing the $[Ca^{2+}]_{cyt}$ response in Arabidopsis roots, and what is the mutated gene in the *cycam1* mutant.

Several exudated compounds have been postulated to induce pathogenicity in plants. Klosterman et al. [3] proposed that based on the sequence information of Verticillium species, pathogenicity may be caused by a cocktail of different compounds and elicitors with different functions in the complex pathogenicity procedure. A Verticillium crude toxin preparation has been often used, although the exact composition of this preparation and the role of the individual compounds are not clear. For instance, recently Yao et al. [59] have demonstrated that the *Vd* toxin preparation stimulates nitric oxide production in Arabidopsis which serves as a signaling intermediate downstream of H_2O_2 to modulate dynamic microtubule cytoskeleton. This may link the *Vd* toxin function again to GLR2.4, who's mRNA level is upregulated after *Vd* infection (Figure 5). Wang et al. [60] reported on the purification and characterization of a novel hypersensitive-like response-inducible protein elicitor named PevD1 from *Vd* that induces resistance responses in tobacco. The relationship of the bioactive compound that induces the $[Ca^{2+}]_{cyt}$ response to the toxins which induce disease responses needs to be investigated.

Interestingly, we did not observe a linear relationship between the propagation of *Vd* in the seedlings and the accumulation of defense-related phytohormone levels. For instance, the phytohormone levels were always high when the seedlings were exposed to *Vd*, irrespective of whether they were exposed to *Vd* alone, pretreated with *Pi* or first with *Vd* followed by *Pi* (Figure 6), although, growth of *Vd* was strongly reduced by the *Pi* pretreatment (Figure 1). This suggests that even low infection rates of *Vd* are sufficient to stimulate the accumulation of the defense hormones. This might be a precaution, although propagation of *Vd* is inhibited when the roots were pretreated with *Pi*.

Various reports showed the involvement of plant hormones in the control of Verticillium growth in Arabidopsis. Stabilization of cytokinin levels enhances Arabidopsis resistance against *V. longisporum* [17]. The fungus also requires JA-dependent COI1 function in roots to elicit disease symptoms in Arabidopsis shoots [15]. Ethylene perception *via* the receptor ETR1 is required for *Vd* infection in Arabidopsis [11]. Enhanced resistance of *etr1-1* plants, but not of SA-, JA- or other ET-deficient mutants against *Vd* infection indicate a crucial role of ETR1 in defense against this pathogen. We observed a particularly striking resistance of the Arabidopsis *ein3* mutant against *Vd* infection *in vivo* and *in vitro* (Additional file 1: Figure S5). This is consistent with the reports by Pantelides et al. [11] for *etr1*, although they did not observe a significant role of EIN3 in their studies. Our data demonstrate that EIN3 plays an important role in

pathogenicity and will provide an important tool to identify EIN3-regulated genes which are required for *Vd* disease development. Furthermore, the ET level in the *ein3* mutant exposed to *Vd* is much higher compared to *Vd*-exposed WT seedlings (Figure 10C). This suggests a feedback loop by which the lack of EIN3-induced defense responses in the *ein3* mutant results in an additional stimulation of ET synthesis.

Conclusions

In summary, our data demonstrate that *Pi* is a very efficient biocontrol agent for *Vd*. This is mainly caused by the restriction of *Vd* growth in the presence of *Pi*. There appears to be additional mechanisms which prevent pathogenicity of *Vd* in the presence of *Pi*. For instance, the phytohormone levels accumulate to comparable levels in *Vd* and *1P2V* seedlings, although *Vd* propagation is restricted in the presence of *Pi* (Figure 1). Since *Pi* pretreatment severely reduces defense gene expression in spite of a comparable phytohormone level in these tissues, additional signals from *Pi* must participate in the regulation of the immune response against *Vd*.

Methods

Growth conditions of seedlings and fungi

A. thaliana wild-type (ecotype Columbia-0) seeds, seeds of the *glr2.4*, *glr2.5*, *glr3.3* and *ein3* mutants as well as of *cycam1* mutant [45] were surface-sterilized and placed on Petri dishes with MS media [61]. After cold treatment at 4°C for 48 h, plates were incubated for 11 days at 22°C under long day conditions (16 h light/8 h dark; 80 μmol m^{-2} sec^{-1}). *Pi* was grown for 3-4 weeks on KM medium as described previously [62]. For detailed information see Section A and B in Johnson et al. [63]. *Vd* (FSU-343, Jena Microbial Resource Center, Germany) was grown for 2-3 weeks on Potato Dextrose Agar (PDA) medium [64].

Co-cultivation assays

For co-cultivation assays 13 day-old *A. thaliana* seedlings of equal size were used. Co-cultivation of *A. thaliana* and the fungi *Pi* and/or *Vd* was performed under *in vitro* culture conditions on a nylon membrane on PNM media as described by Johnson et al. ([63], Section C1 - Method 1) with a few modifications. *Vd* was grown for 12 days and *Pi* for 10 days on the membrane on top of PNM medium in Petri dishes. 13-day old Arabidopsis seedlings were then transferred to the *Pi* or *Vd* plates, or mock-treated (no fungal mycelium; C). For the shifting experiments, the seedlings were transferred to plates with the other fungus after 4 days (from *Vd* to *Pi* or *vice-versa*). Including the (C), five different treatments were compared: (1) Arabidopsis seedlings grown without *Pi* or *Vd* (C); (2) without *Pi* and with *Vd* (*Vd*); (3) with *Pi* and without *Vd* (*Pi*); (4) with *Pi* for 4 days before transfer to *Vd* plates (*1P2V*) and (5) with *Vd* for 4 days before transfer to *Pi* plates (*1V2P*). The seedlings were harvested between 1 and 21 days after exposure to the first fungus (or mock-treatment) for further analysis. A time scheme is shown in Additional file 1: Figure S1. The light intensity (80 μmol m^{-2} s^{-1}) was checked weekly. Shoots and roots were harvested separately for DNA and RNA analyses.

Long term co-cultivation in sterile vermiculite

30 g vermiculite was placed into one Magenta box (Sigma-Aldrich, Germany) and autoclaved at 121°C for 30 min. After the addition of 40 ml of sterile liquid PNM medium, Arabidopsis seedlings grown in Petri dishes for 10 days were transferred to the sterile vermiculite boxes (1 plant per box). For each treatment, 16 seedlings were analyzed. After 10 days, the number of survived plants, their biomass and fungal DNA content were determined.

Gene expression analysis

RNA was isolated from shoots and reverse-transcribed for Real-time quantitative PCR analysis, using an iCycler iQ Real-time PCR detection system and iCycler software version 2.2 (Bio-Rad). Total RNA was isolated from 5 independent biological experiments of Arabidopsis shoots. cDNA was synthesized using the Omniscript cDNA synthesis kit (QIAGEN) using 1 μg RNA. For the amplification of the RT-PCR products, iQ SYBR Green Supermix (Bio-Rad) was used according to the manufacturer's protocol in a final volume of 20 μl. The iCycler was programmed to 95°C 3 min, 40 × (95°C 30 sec, 57°C 15 sec, 72°C 30 sec), 72°C 10 min, followed by a melting curve program 55°C to 95°C in increasing steps of 0.5°C. All reactions were performed in triplicate. The mRNA levels for each cDNA probe were normalized with respect to the glycerin-aldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA level. The primer pairs are given in Additional file 1: Table S1.

Quantification of fungal DNAs by PCR

Genomic DNA extraction was conducted with DNeasy Plant Mini Kit. 12.5 ng DNA was taken for PCR template. The reactions were performed with gene-specific primers, as given in Additional file 1: Table S1. For details see Camehl et al. [65].

Dual culture of *Pi* and *Vd*

Dual culture of *Pi* and *Vd* on agar plates was performed as described by Johnson et al. [66]. A *Pi* plug with 5 mm diameter was placed at one end of a PDA plate and a *Vd* plug of the same size at the other end of the plate. The plates were incubated at 22-24°C in dark and 75%

relative humidity. Photos were taken after 3 weeks of co-cultivation.

Percentage disease index (PDI) calculation

Disease index was calculated with the following formula:

$$PDI = \frac{n_1x_1 + n_2x_2 + n_3x_3 + n_4x_4 + n_5x_5}{\text{Total number of leaves observed} \times \text{maximum grade}} \times 100$$

$n_{1\text{-}5}$ = number of affected leaves of the respective disease.

Severity grade (0-5), $x_{1\text{-}5}$ = disease severity grade based on the percentage of affected leaf area. 1, $1\% \leq x \leq 10\%$; 2, $10\% < x \leq 20\%$; 3, $20\% < x \leq 30\%$; 4, $30\% < x \leq 40\%$; 5, $x > 40\%$; ×100: calculated in percentage scale. Disease severity was estimated on the basis of affected leaf area. 1-5 disease severity grades were described by Naik and Lakkund [67,68].

Quantification of jasmonic acid (JA), JA-isoleucine (JA-Ile), abscisic acid (ABA), salicylic acid (SA), oxophytodinoic acid (OPDA) and ethylene (ET)

Independent samples of 250 mg shoot material were collected from each treatment. Phytohormone extractions (JA, JA-Ile, ABA, SA and OPDA) were performed by adding 1 ml ethyl-acetate containing 60 ng of D_2-JA and 40 ng of D_6-ABA, D_4-SA and JA-$^{13}C_6$-Ile (OPDA has the same internal standerd as JA) to 100 mg ground tissues. All samples were then vortexed for 10 min and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatants were collected and evaporated to dryness at 30°C using a vacuum concentrator. Residues were resuspended in 500 μl MeOH:H_2O (70:30, v/v) and centrifuged at 13,000 rpm for 10 min. The supernatants were collected and measured with the API 3200 LC-MS/MS system (Applied Biosystems, Framingham, USA) as previously described [69].

For ET measurements, 100 mg shoot material from each treatment was collected into 4 ml vials (Roth, Germany). After 4 h ET accumulation, the measurement was performed with the ETD-300 ethylene detector (Sensor Sense B.V., Nijmegen, The Netherlands).

Chlorophyll content was determined according to Yang et al. [70] and based on g fresh weight.

Quantification of microsclerotia

Roots of Arabidopsis seedlings from the 3 treatments with *Vd* were harvested after 3 weeks of co-cultivation in Petri dishes and transferred to a microscopic glass slide with 80 μl lactic acid/glycerol/H_2O (1:1:1). The number of the microsclerotia formed in the roots was calculated averagely per root visually under the light microscope (magnification: x200). The experiment was performed 3 times independently and for each treatment the roots of 12 seedlings were analysed.

Cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) measurement

Aequorin based luminescence measurements were performed using 21-day old individual wild-type (WT) plants and mutants grown in Hoagland medium [71]. WT aequorin (pMAQ2) plants served as control [72]. Mutants (*glr2.4*, *glr2.5* and *glr3.3*) were crossed back to wild-type expressing aequorin. After 2 generation selection based on $[Ca^{2+}]_{cyt}$ responses and RT-PCR of T-DNA insertion examination, the homozygote seeds were used for the described experiments. Primers used for homozygosity tests are given in Additional file 1: Table S1. For $[Ca^{2+}]_{cyt}$ measurements, approximately 70% of the roots per seedling was dissected and incubated overnight in 150 μl of 7.5 μM coelentrazine (P.J.K. GmbH, Germany) in the dark at 20°C in a 96 well plate (Thermo Fischer Scientific, Finland, cat. no. 9502887). Bioluminescence counts from roots were recorded as relative light units (RLU) with a microplate luminometer (Luminoskan Ascent, version 2.4, Thermo Electro Corporation, Finland).

Preparation of exudates from mycelia of *Vd*

A 5 mm *Vd* fungal plug was inoculated in Czapek's medium as described in Zhen et al. [73] and grown for 3 weeks. Then, the fungal culture was filtered through double layers of filter paper and the filtrate was centrifuged at 10,000 *g* for 30 min to remove the spores. The supernatant was dialyzed with a dialysis membrane (MWCO) (Spectra/Por® Float-A-lyzer®) in 10 mM phosphate buffer pH 7.0 at 4°C for 24 h. The dialyzed solution was frozen and lyophilized. The powder was dissolved in distilled water and the solution was filtered through a 0.45 μm pore size Millipore filter (Roth, Germany). The resulting filtrate was used as exudate for further experiments.

Statistics

All statistical analyses were performed using Excel or SPSS 17.0 (SPSS Inc., Chicago, IL, USA) for ANOVA.

Availability of supporting data

All the supporting data are included as additional file.

Additional file

Additional file 1: Figure S1. Co-cultivation time scheme. The seeds were first kept at 4°C in the dark for 2 days and were then transferred to a light/dark cycle at 22°C for 9 days. These seedlings were used for the experiments, by either transferring them to a plate with *Vd* or *Pi* (or no fungus, control, C) at day 0. The seedlings were harvested 10, 14 or 21 days later. In case of transfer from *Vd* to *Pi* or *vice versa*, the transfer occurred at day 4. **Figure S2.** Induction of *GLR* genes in shoots of Arabidopsis seedlings after 1 and 14 days. **Figure S3.** Phenotype of *ein3-1* and WT after 21 days of co-cultivation following the 5 treatments described in Methods. **Figure S4.** ET content in shoots of *ein3-1* seedlings after 3 weeks. **Figure S5.** Phenotypes of WT and *ein3-1* after *Vd* spore inoculation *in vivo* and *in vitro*.

Figure S6. Phenotype of WT and *cycam1* mutant 21 days after *Vd* inoculation. **Table S1.** Primer list for RT-PCR and PCR analysis.

Abbreviations

Vd: *Verticillium dahliae*; *Pi*: *Piriformospora indica*; $[Ca^{2+}]_{cyt}$: Cytosolic calcium; *cycam1*: *Cytosolic calcium elevation mutant1*; *glr*: *Glutamate receptor* mutants; *ein3*: *Ethylene-insensitive3 mutant*; JA: Jasmonic acid; JA-Ile: Jasmonyl-isoleucine; ABA: Abscisic acid; SA: Salicylic acid; OPDA: Oxophytodinoic acid; ET: Ethylene; WT: Wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CS designed and carried out most of the experiments. YQS prepared the exudates from *V. dahliae*. KV helped for root microscopy and long term experiments in soil. JL and SB did the phytohormone analysis. SD, K-WY, BL and ITB contributed to the discussion. CS, IS and RO wrote the article. RO supervised the research. All authors read and approved the final manuscript.

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4.5 Manuscript V

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Balancing defense and growth—Analyses of the beneficial symbiosis between *Piriformospora indica* and *Arabidopsis thaliana*

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Abstract The mutualistic interaction between the endophytic and root-colonizing fungus *Piriformospora indica* and *Arabidopsis thaliana* is a nice model system to study beneficial and non-benefical traits in a symbiosis. Colonized Arabidopsis plants are taller, produce more seeds and are more resistant against biotic and abiotic stress. Based on genetic, molecular and cellular analyses, Arabidopsis mutants were identified which are impaired in their beneficial response to the fungus. Several mutants are smaller rather than bigger in the presence of the fungus and are defective in defense responses. This includes mutants with defects in defense-signaling components, defense proteins and enzymes, and defense metabolites. The mutants cannot control root colonization and are often over-colonized by *P. indica*. As a consequence, the benefits for the plants are lost and they try to restrict root colonization by activating unspecific defense responses against *P. indica*. These observations raise the question as to how the plants balance defense gene activation or development and what signaling molecules are involved. *P. indica* promotes the synthesis of phosphatidic acid (PA), which binds to the 3-PHOSPHOINOSITIDE-DEPENDENT-KINASE1 (PDK1). This activates a kinase pathway which might be crucial for balancing defense and growth responses. The review describes plant defense compounds which are necessary for the mutualistic interaction between the two symbionts. Furthermore, it is proposed that the PA/PDK1 pathway may be crucial for balancing defense responses and growth stimulation during the interaction with *P. indica*.

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1 Introduction

Mutualistic interaction is a type of symbiosis in which two partners benefit from each other. Mycorrhizae are a classical example: the fungus delivers soil nutrients to the plant and the plant supplies the fungus with carbon compounds. We studied the mutualistic interaction between a root colonizing endophyte, *Piriformospora indica*, and the model plant *Arabidopsis thaliana* (cf. Johnson and Oelmüller 2009). *P. indica*, a cultivable basidiomycete of Sebacinales, colonizes the roots of many plant species including Arabidopsis (Peškan-Berghöfer et al. 2004; Oelmüller et al. 2009; Qiang et al. 2012; Reitz et al. 2012; Lahrmann and Zuccaro 2012). Like other members of Sebacinales, *P. indica* is found worldwide in association with roots (Selosse et al. 2009) and stimulates growth, biomass and seed production of the hosts (Peskan-Berghöfer et al. 2004; Oelmüller et al. 2009; Shahollari et al. 2007; Sherameti et al. 2005, 2008a and b; Vadassery et al. 2009a and b; Waller et al. 2005; Zuccaro et al. 2011). The fungus promotes nitrate and phosphate uptake and metabolism (Sherameti et al. 2005; Shahollari et al. 2004; Yadav et al. 2010). *P. indica* also confers resistance against abiotic (Sherameti et al. 2008a; Baltruschat et al. 2008; Sun et al. 2010) and biotic stress (Oelmüller et al. 2009; Stein et al. 2008). The broad host range of *P. indica* indicates that the beneficial interaction may be based on general recognition and signaling pathways. Enhanced plant growth can be induced by an fungal exudate component (Vadassery et al. 2009a), suggesting the involvement of specific receptors at the plant cell surface. In support of this hypothesis, an atypical receptor kinase with leucine-rich repeats was identified as being required for the growth response in Arabidopsis (Shahollari et al. 2007).

Moreover, a rapid increase in the intracellular calcium concentration in the root cells indicates that an intracellular signaling cascade is triggered early upon plant-fungal interaction (Vadassery et al. 2009a).

Here, one class of mutants is described, for which the interaction is no longer beneficial for the plant. While growth and performance of wild-type plants is promoted by the fungus, colonized mutants are smaller in the presence of the fungus. They produce less seeds and biomass and normally grow slower. These mutants have defects in different and unrelated defense responses, i.e. either in signaling molecules or transcription factors which activate defense genes, or in genes for enzymes which are required for the synthesis of defense compounds. All these mutants have in common that they cannot control root colonization by *P. indica*. Their roots are overcolonized, consequently, the plants show stress symptoms and express stress-related genes. The overcolonized roots try to restrict root colonization by upregulating defense genes which are not impaired by the mutations. Thus, a mild and constitutive defense response is required for establishing or maintaining a beneficial symbiosis between the two partners. Interestingly, the mutated genes code for enzymes involved in quite different and unrelated defense processes. How do the plants balance defense gene activation and development, and how do they distinguish between friends and foes (cf. Johnson and Oelmüller 2009; Paszkowski 2006; Kogel et al. 2006; Tunlid and Talbot 2002)? A model is proposed that describes a balanced activation of defense and growth / development depending on the environment.

2 Ethylene signaling is required for the beneficial interaction between *P. indica* and Arabidopsis

Mutants defective in the ethylene signaling components ETR1 and EIN2 and the ethylene-targeted transcription factors EIN3/EIL1 are unable to establish a beneficial interaction with *P. indica* (Camehl and Oelmüller 2010). Ethylene is perceived by a family of endoplasmatic reticulum-associated two component kinases, one of them is ETR1. The hormone binds to this receptor via a copper co-factor, which results in the inactivation of the receptor function (Hua and Meyerowitz 1998).

ETR1, EIN2 and EIN3/EIL1 are required for *P. indica*-mediated growth promotion of Arabidopsis seedlings (Camehl et al. 2010). Growth promotion by *P. indica* of the corresponding single (*etr1*, *ein2*) and double (*ein3 eil1*) knockout lines is impaired. Therefore, these ethylene-related genes participate in balancing beneficial and non-beneficial traits in the symbiosis. The signaling compounds are also required for restricting growth of the fungus in the roots, by activating defense genes and other defense responses. The mutant roots are overcolonized which is harmful for the plants. This hypothesis is further supported by the observation that ERF1 overexpressors, which show constitutively activated defense responses, are less colonized. Apparently, manipulation of ethylene-induced defense responses has a strong influence on the degree of root colonization, which in turn determines whether the symbiotic interaction is beneficial or harmful. The fungus does not induce these ethylene-dependent signaling compounds at the transcriptional level, as observed after pathogen infections. It appears that the available amount of these signaling components is sufficient to establish a mild defense response for the restriction of root colonization.

A. thaliana contains 147 ERF (ethylene-responsive element-binding factor) transcription factors with mostly uncharacterized functions. Two of them, ERF9 and ERF14 have been investigated in more details because their mRNA levels are upregulated during early phases of the symbiotic interaction between *P. indica* and Arabidopsis roots. Insertional inactivation of the two genes *ERF9* and *ERF14* has a negative effect on the beneficial interaction between the two symbionts. The mutants are diminished in *P. indica*-induced growth promotion and activate the expression of the *PATHOGENESIS-RELATED1* and *−2* genes. This and additional observation (Camehl and Oelmüller 2010) led to the conclusion that ERF9 and ERF14 represses *PR* gene expression in colonized Arabidopsis roots and that this contributes to the establishment of the beneficial interaction.

Taken together, ethylene signaling components and ethylene-targeted transcription factors are required for restriction of root colonization in wild-type seedlings and adult plants. Since ERF transcription factors can function as transcriptional activators and repressors, they are candidates for establishing a balanced defense response to the fungus without preventing growth and development.

3 WRKY transcription factors are targets of *P. indica* in Arabidopsis roots and leaves

The WRKY transcription factor family plays an important role in the regulation of transcriptional reprogramming of the plants in response to abiotic (Chen et al. 2012) and biotic (Pandey and Somssich 2009) stress. They are involved in various aspects of plant/microbe interactions and plant immunity (Pandey and Somssich 2009). This huge gene family forms a regulatory network, in which the individual members participate in quite different stress responses. In a similar way to the ERFs, they function as positive and negative regulators of gene expression and form complex protein-protein interactions. They interact with MAP kinases, MAP kinase kinases, 14-3-3 proteins, calmodulin, histone deacetylases, resistance proteins and other WRKY transcription factors (Rushton et al. 2010). Most of the studies to date have been performed with leaf tissue, while the role of WRKYs in the roots has been less investigated. WRKY transcription factors also play a central role in controlling leaf senescence in Arabidopsis. One member of this family,

WRKY53, is tightly regulated by unexpected mechanisms and is a convergence node between senescence and biotic and abiotic stress responses (Zentgraf et al. 2010). Interestingly, the *WRKY53* mRNA level is strongly regulated by *P. indica* in Arabidopsis roots (Table 1). As in ERFs, the WRKYs provide another example of a transcription factor family that can integrate diverse internal and environmental signals which allows a rapid and dynamic response to changing environmental conditions. Table 1 presents a summary of the regulation of *WRKY* transcription factor genes in the roots of Arabidopsis seedlings after 2 and 6 days of co-cultivation with *P. indica*. The relatively large number of *WRKY* genes which are differentially regulated in Arabidopsis roots after co-cultivation with *P. indica* suggests that they play a crucial role in the symbiosis. The role of these transcription factor genes in the symbiotic interaction is currently under study.

4 *Cerk* mutants

A fast method for testing root colonization was set up, which allows also the quantification of root colonization (in contrast to methods described previously; cf. McGonigle et al. 1990). The seedlings were kept on PNM medium (Johnson et al. 2011) in the presence of *P. indica* for 14 days. The roots were removed and stained on a glass slide with 100 µl Nile red stain solution (0.005 % Nile red in 75 % glycerol) for 10 min. Microscopy was performed with a Zeiss Oxiovert 135 instrument under the fluorescent channel at 450–520 nm. This staining method results in a high contrast between plant tissue and fungal spores (Figs. 1 and 2) and hyphae (Fig. 2c, d). They can be easily visualized and quantified with the Adobe PhotoshopTM software, by counting pixel ratios. The amount of fungal material can be related to the root area (Fig. 1a, b) or to the root length (Fig. 1c, d). The distribution of fungal material in the entire root is analysed at lower microscopic resolution. Representative sections from different regions of the roots were then analysed in more details to obtain quantitative data. Root colonization is subsequently confirmed by molecular markers, by which the *P. indica TRANSLATION ELONGATION FACTOR1* mRNA or DNA levels are expressed relative to the amount of the plant *GAPC2* mRNA or DNA levels (Bütehorn et al. 2000; Camehl et al. 2011). Although we have not observed many differences between the staining methods and the molecular method, the staining method is faster and allows the localization of the spores and hyphae in the root.

Table 1 Regulation of *WRKY* genes in the roots of Arabidopsis seedlings co-cultivated by *P. indica* for 2 or 6 days on agar plates (cf. Johnson et al. 2011). Based on 3 independent microarray analyses, the values represent fold induction relative to the mock-treated control and are average values of the three hybridizations. The list of the WRKY family members was taken from http://www.arabidopsis.org/browse/genefamily/WRKY-Som.jsp. Only those genes are shown which are regulated > 2-fold at one time point

Protein Name	Genome Locus	TIGR annotation	2 days co-cultivation with *P. indica*	6 days co-cultivation with *P. indica*
Group I				
WRKY25	At2g30250	putative WRKY-type DNA binding protein	2.2 (± 0,31)**	1.7 (± 0,29)**
WRKY33	At2g38470	putative WRKY-type DNA binding protein	5.9 (± 0,98)**	2.9 (± 0,37)**
WRKY45	At3g01970	putative WRKY-like transcriptional regulator protein	4.8 (± 0,88)**	1.2 (± 0,26)
Group II-a				
WRKY40	At1g80840	transcription factor, putative	4.5 (± 0,91)**	0.9 (± 0,15)
WRKY60	At2g25000	putative WRKY-type DNA binding protein	0.4 (± 0,10)**	1.1 (± 0,14)
WRKY6	At1g62300	unknown protein	4.4 (± 0,79)**	1.0 (± 0,19)
Group II-b				
WRKY9	At1g68150	putative DNA binding protein	0.4 (± 0,33)**	1.0 (± 0,17)
WRKY31	At4g22070	putative protein	3.3 (± 0,61)**	2.4 (± 0,42)
WRKY61	At1g18860	hypothetical protein	2.6 (± 0,52)**	1.6 (± 0,33)
Group II-e				
WRKY14	At1g30650	putative DNA-binding protein	0.5 (± 0,11)	1.1 (± 0,23)
Group III				
WRKY38	At5g22570	putative protein	2.0 (± 0,43)	4.1 (± 0,55)**
WRKY53	At4g23810	putative protein	5.3 (± 1,02)**	2.0 (± 0,44)**
WRKY54	At2g40750	hypothetical protein	4.0 (± 0,79)**	5.1 (± 0,96)**
WRKY70	At3g56400	DNA-binding protein-like	4.2 (± 0,80)**	5.0 (± 1,16)**

Errors were calculated as standard errors. Relative errors of the proportion are the sum of the individual relative errors. **, significantly different from the uncolonized control ($p<0.05$)

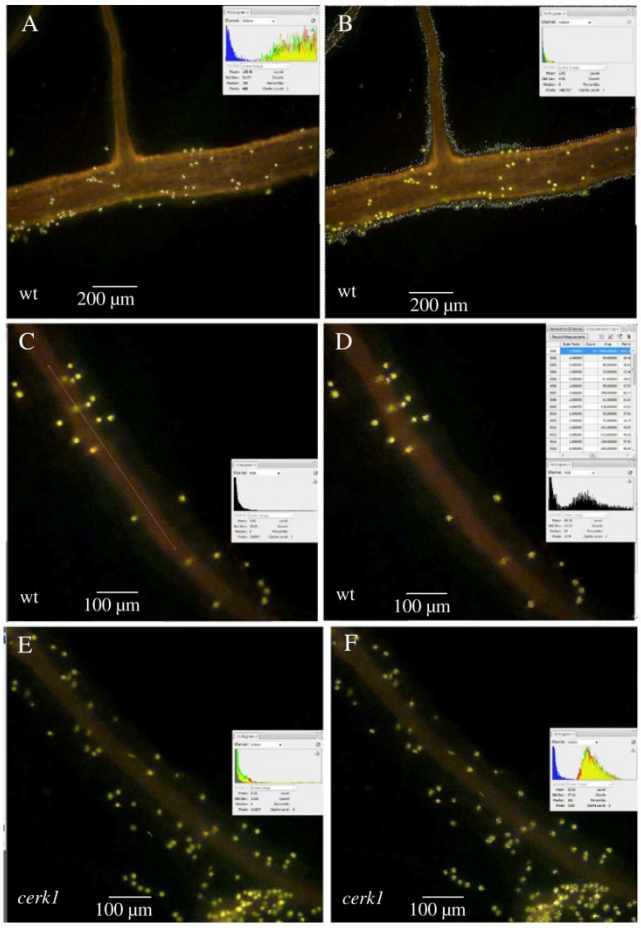

Fig. 1 Root colonization of wild-type (**a**, **b**, **c**, **d**) and *cerk1* (**e**, **f**) seedling grown on PNM media for 2 weeks. Root area was measured by Magic wand tool adjusted with tolerance of 32 for each sample (**b**). The colonization pixels were selected by Adobe Photoshop CS5 Magic wand tool adjusted with tolerance of 50. The signals of the selected pixels were quantified by the Histogram tool (**a**, **d**, **f**). The length of root was measured with the Photoshop Ruler Tool for each sample (**c**). Number of selected spores is available in Measurement Log window (**d**). Spore/root area ratio was calculated on the basis of the whole root. Only wild type and mutant roots of equal size were considered. Root colonization was calculated on the basis of the spore selected pixels relative to root area as [selected pixel/root area]×1000 and root length as [selected pixel/root length]

Using these methods CERK1 was identified as an important component for the beneficial interaction between the two symbionts. CERK1 is activated by chitin, which is the main component of the cell walls of beneficial and pathogenic fungi. Chitin fragments are recognized by plant lysin motif (LysM)-containing proteins, which, in case of pathogens, activate signaling events leading to innate immunity. In Arabidopsis; CERK1 is one of the first LysM-containing receptor-like kinase 1 (LYK1) which was identified as a chitin recognizing protein (Miya et al. 2007; Wan et al. 2008). In rice (*Oryza sativa*) the LysM-containing protein "chitin elicitor binding protein" (CEBiP) is involved in chitin recognition (Kaku et al. 2006). Arabidopsis possesses three *CEBiP*-like genes and five *LYK* genes. Inactivation of *CERK1* results in a reduced induction of chitin-responsive genes (cf. Wan et al. 2012 and references therein for the original publications). *cerk1* is overcolonized by *P. indica*, which has also been demonstrated by Jacobs et al. (2011). Under the assumption that CERK1 in the beneficial *P. indica*/Arabidopsis interaction has a similar function to that in pathogenic interactions, chitin or related compounds from *P. indica* should activate CERK1-dependent defense processes in Arabidopsis roots. *P. indica* might induce a mild defense response via CERK1 activation and this represents an additional facet in the restriction of root colonization.

Microarray analyses suggest that *CERK* and *CEBiP*-like genes are barely regulated by *P. indica* (Table 2). The strongest response was shown for *CERK4*. Therefore, besides CERK1, CERK4 might also be involved in restricting root colonization in Arabidopsis roots (cf. Wan et al. 2012).

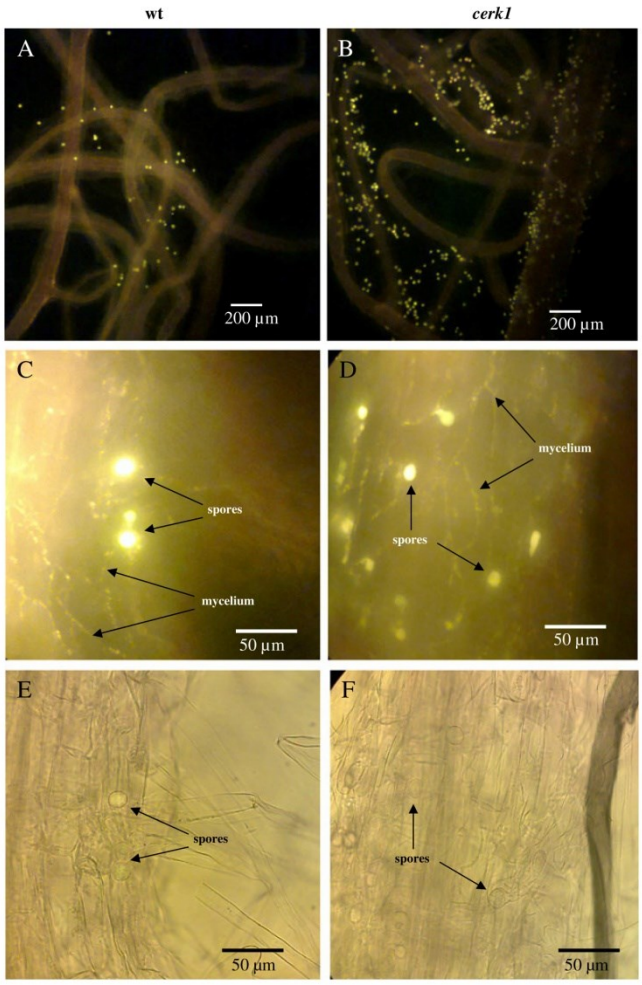

Fig. 2 Root colonization of Arabidopsis wild-type (*left*) and *cerk1* line (*right*). The fungus was stained with Nile red and monitored under the fluorescent channel at 450–520 nm (**a**–**d**) and under visual light (**e**, **f**). Comparison of fluorescent and visual light microscopy in discrimination of spores and mycelium in wt (**c**, **e**) and *cerk1* roots (**d**, **f**)

5 Glucosinolates and enzymes of the glucosinolate metabolism are required to establish or maintain a mutualistic interaction between *P. indica* and Arabidopsis

Members of the order Brassicales synthesize important secondary metabolites such as glucosinolates from tryptophan and methionine. This group of compounds with over 120 different identified chemical structures (Fahey et al. 2001; Sønderby et al. 2010; Janowitz et al. 2009; Piotrowski 2008) and their degradation products provide protection against insect heribivory (McCloskey and Isman 1993; Giamoustaris and Mithen 1995; Müller et al. 2010). The constitutive production of phytoanticipins or phytoalexin is important for plant defense against microbes (Hammerschmidt 1999; Pedras et al. 2007; Bednarek and Osbourn 2009). Upon attack by necrotrophic fungi, Arabidopsis induces the synthesis of the phytoalexin camalexin (Schuhegger et al. 2006; Ferrari et al. 2003). CYP79B2 and CYP79B3 are two functionally redundant cytochrome P450 enzymes which convert tryptophan into indole-3-acetaldoxime (IAOx). This is an intermediate for the biosynthesis of indole glucosinolates (I-GLS), camalexin, other indole compounds such as indole acetonitrile, indole carboxylic acid derivatives, and, under specific conditions, the plant hormone indole-3-acetic acid (IAA). The double *cyp79B2 cyp79B3* mutant lacks I-GLS (Zhao et al. 2002)

Table 2 Fold-induction of the mRNA level for CEBiP and CERK proteins in colonized Arabidopsis roots relative to the mock-treated uncolonized control. Co-cultivation with *P. indica* was performed for 2 or 6 days. Based on 3 independent microarray analyses, the data are averages of the three experiments

Protein Name	Genome Locus	2 days co-cultivation	6 days co-cultivation
CEBiP-like1	At2g17170	n.d.	n.d.
CEBiP-like2	At1g21880	1.01 (± 0,18)	1.31 (± 0,20)
CEBiP-like3	At1g77630	0.96 (± 0,16)	1.33 (± 0,17)
CERK1	At3g21630	1.32 (± 0,22)	1.22 (± 0,20)
CERK2	At3g01840	n.d.	n.d.
CERK3	At1g51940	0.87 (± 0,11)	0.65 (± 0,09)
CERK4	At2g23770	1.94 (± 0,23)	2.22 (± 0,29)
CERK5	At2g33580	0.89 (± 0,13)	1.22 (± 0,21)

Errors were calculated as standard errors. Relative errors of the proportion are the sum of the individual relative errors. Only the CERK4 values 2 and 6 days after co-cultivation are significantly different from the uncolonized control ($p<0.05$). *n.d.*, not detectable

and is unable to induce camalexin synthesis (Glawischnig et al. 2004). Furthermore, it does not accumulate indole-3-carboxylic acid derivatives (Böttcher et al. 2009), i.e. secondary metabolites which are strongly induced by pathogen infections. *P. indica* colonization causes severe growth defects on agar plate-grown *cyp79B2 cyp79B3* seedlings as well as adult plants in soil (Nongbri et al. 2012). This demonstrates that IAOx-derived compounds are essential in the beneficial interaction between Arabidopsis and *P. indica*. PAD3, the last enzyme of camalexin biosynthetic pathway is regulated by a variety of signaling components such as the mitogen-activated protein kinases (MPK) MPK3, MPK6 (Ren et al. 2008) and MPK4 (Qiu et al. 2008). Co-cultivation of Arabidopsis seedlings with *P. indica* on agar plates induced significantly higher levels of camalexin in the roots compared to mock-treated controls (Nongbri et al. 2012). The mRNA levels for CYP79B2, CYP79B3, CYP71A13 (Nafisi et al. 2007), PAD3, and WRK33 (Qiu et al. 2008) are upregulated in colonized wild-type (WT) roots, whereas those for CYP83B1 and SUR1 are not (Nongbri et al. 2012). This demonstrates that the genes for the synthesis of IAOx-derived compounds, including camalexin but not I-GLS, are targets of signals from the fungus. In contrast to the *cyp79B2 cyp79B3* double mutant which is impaired in *P. indica*-mediated growth promotion at seedling and adult stage, the *pad3* mutant is not affected during the initial stage of interaction. However, since growth of adult *pad3* plants is not promoted by *P. indica*, camalexin plays an important role during long term interaction (Nongbri et al. 2012).

5.1 PEN2 (At2g44490)

Screening for Arabidopsis mutants deficient in resistance to barley powdery mildew identified *penetration* (*pen*) mutants. The *PEN2* gene encodes a glycosyl hydrolase which restricts pathogen entry of two powdery mildew fungi into Arabidopsis leaf cells (Lipka et al. 2005). PEN2 localizes to the peroxisomes and acts as a component of an inducible preinvasion resistance mechanism. The *pen3* plants permitted both increased invasion into epidermal cells and initiation of hyphae by *B. hordei*, suggesting that PEN3 contributes to defenses at the cell wall and intracellularly. PEN3 may be involved in exporting toxic materials to attempted invasion sites.

Microarray analysis with *P. indica*-colonized vs. uncolonized Arabidopsis roots demonstrated that all *PEN* genes are expressed in roots and slightly upregulated in response to *P. indica* (Table 3). The strongest response was observed for *PEN2*. A knock-out mutant (kindly obtained from Prof. Schulze-Lefert, MPI Cologne) for PEN2 also showed severe overcolonization of the roots and does not respond properly to the fungus (Seebald et al. unpublished). Similar results have been reported by Jacobs et al. (2011). This indicates that PEN2 participates in the restriction of root colonization and suggests that general mechanisms restrict colonization of plant cells, irrespective of whether they are colonized by pathogens or beneficial microbes. The role of PEN1 and PEN3 is currently under study, however their mRNA levels respond less to *P. indica* colonization in Arabidopsis roots when compared to that for PEN2 (Table 3).

5.2 Pyk10

PYK10 is an abundant protein in the roots of Brassicaceae. Although it appears to be a ß-glucosidases or myrosinases, an enzymatic activity for this protein has not yet been demonstrated. The role of PYK10 in beneficial and pathogenic plant/microbe interactions is not clear. In general, mycrosinases hydrolyze ß-glucosidic bonds of aryl ß-D-glucosides, as well as ß-glucosides with carbohydrate moieties such as cellobiose and other ß-linked oligosaccharides. In particular, the enzymes hydrolyze non-toxic glucosinolates to biologically

Table 3 Fold-induction of the mRNA level for PEN proteins in colonized Arabidopsis roots relative to the uncolonized control. Co-cultivation was performed for 2 or 6 days. Average values based on 3 independent microarray analyses. Errors were calculated as standard errors

Protein Name	Genome Locus	2 days co-cultivation	6 days co-cultivation
PEN1	At3g11820	1.44 (± 0,27)	1.15 (± 0,17)
PEN2	At2g44490	2.25 (± 0,31)	1.07 (± 0,19)
PEN3	At1g59870	1.16 (± 0,22)	1.13 (± 0,14)

Relative errors of the proportion are the sum of the individual relative errors. Only the PEN2 value 2 days after co-cultivation is significantly different from the uncolonized control ($p<0.05$)

active and toxic isothiocyanates, thiocyanates, nitriles and other epithio nitriles and it is believed that the biological function of the myrosinases depends on the nature of the aglycon moieties released from the substrates. To prevent the release of the toxic compounds, myrosinases are present in the endoplasmic reticulum. Release of the enzyme requires damage to the cell. This would mean that the symbiotic interaction between the two symbionts studied here results, at least in part, in cell damage. Alternatively, a minor fraction of the highly abundant protein might also be released from the endoplasmic reticulum due to naturally occurring cell death. This minor fraction of PYK10 might be sufficient to release toxic compounds from conjugates and therefore participates in restriction of root colonization. Since the substrate of PYK10 is not known at present, another explanation might be that the enzyme has an additional function in the cell or that the highly abundant protein catalyzes unspecific and unknown site reactions, which results in the generation of toxic compounds which restrict fungal growth and thus root colonization.

PYK10 is required for the beneficial interaction between Arabidopsis and *P. indica* (Sherameti et al. 2008b). Insertional inactivation of *PYK10* in Arabidopsis results in the loss of the benefits for the plants when the roots are colonized by *P. indica*: growth promotion is no longer visible and for adult plants, the seed production is not enhanced (Sherameti et al. 2008b). Expression of *PYK10* is controlled by the helix-loop-helix containing transcription factor NAI1 and inactivation of this transcription factor gene results in a severe reduction of *PYK10* gene expression. The *nai1* mutant behaves like the *pyk10* mutant in response to the fungus, which confirms the essential role of the myrosinase for the beneficial interaction. Closer inspection of the roots showed that the degree of colonization is significantly higher compared to the wild-type control. This suggests that PYK10 participates in the restriction of root colonization. Like in other mutants, overcolonization of the roots results in a mild activation of defense genes. In particular *PDF1.2* is a very sensitive defense marker gene which is rapidly upregulated when the mutualistic interaction is no longer balanced. In the overcolonized *pyk10* mutant, *PDF1.2* is strongly upregulated (Sherameti et al. 2008b).

PYK10 shares sequence similarities with other family members. One of them is PEN2. Like PEN2, PYK10 belongs to the class of glycosyl hydrolase family 1, both proteins are located in intracellular organellar structures (PYK10 in ER bodies and PEN2 in peroxisomes), and both proteins share a high degree of sequence similarity. The catalytic domains of both proteins contain two conserved nucleophilic glutamates. Lipka et al. (2005) have shown that glutamate^{183} is required for PEN2 *function* in vivo, which suggests that PEN2 catalytic activity is required for restricting pathogen entry. Thus, PYK10 might have a similar biological function in our system.

The beneficial traits in the *P. indica*/Araidopsis symbiosis are highly dependent on the density of the hyphae in and around the root (Camehl et al. 2011). Increasing quantities of hyphae resulted in a suboptimal interaction. Furthermore, marker genes for the beneficial interaction were downregulated and those for defense processes, such as *PDF1.2*, were upregulated in the roots in a dose-dependent manner (Oelmüller et al. 2009). Similar response patterns were observed for *PYK10* overexpressor and knockout lines (Sherameti et al. 2008b). In order to maintain a mutualistic interaction with benefits for both partners, the degree of root colonization might be controlled by activating PYK10-dependent defense responses, when too many hyphae colonize the roots and the cells become damaged or wounded by hyphal penetration. In barley, for instance, less-defended root cells undergo cell death after colonization with *P. indica* (Deshmukh et al. 2006).

Figures 3 and 4 summarizes identified plant defense responses which are required for the restriction of Arabidopsis root colonization by *P. indica*. These compounds are involved in signal perception (ETR1, CERK1), in plant signal transduction processes such as the MAPKs, in transcriptional activation such as the ethylene transcription factor members EIN3 and EIL1, ERF1, -9 and -14, as well as members of the WRKY family, defense proteins (PEN2 and probably PYK10), as well as defense metabolites (such as camalexin).

6 Induced systemic resistance: beneficial root-colonizing microbes protect the leaves against pathogens

Induced systemic resistance (ISR) is mediated by beneficial soil-borne microorganisms, such as plant growth promoting rhizobacteria, mycorrhizal fungi or beneficial endophytes. They improve plant performance by inducing systemic defense responses that confer broad-spectrum resistance to plant pathogens and even insect herbivores (van Wees et al. 2008). Different beneficial microbe-associated molecular patterns (MAMPs) are recognized by the plant, which results in a mild, but effective activation of the plant's immune responses in systemic tissues. Systemic resistance induced by different beneficial microbes is regulated by jasmonate-dependent and ethylene-dependent signaling pathways and is associated with priming for enhanced defense (van Wees et al. 2008). A large body of evidence for such a regulatory circuit is described in the literature.

When roots of Arabidopsis seedlings are colonized by *P. indica*, the leaves are much more resistance to *Alternaria brassicae* infections compared with the uncolonized control. This clearly demonstrates root to shoot signaling induced by *P. indica* (cf. also Stein et al. 2006) Several ethylene and jasmonic acid signaling mutants were tested, but the protective function of *P. indica* against *A. brassicae* infection was still evident with these mutants. Therefore, ethylene and jasmonic acid signaling play no or only a minor role in *P. indica*-ISR against *A. brassicae*. However, when the *monodehydroascorbate reductase2* (*mdar2*; SALK_0776335C) and *dehydroascorbate*

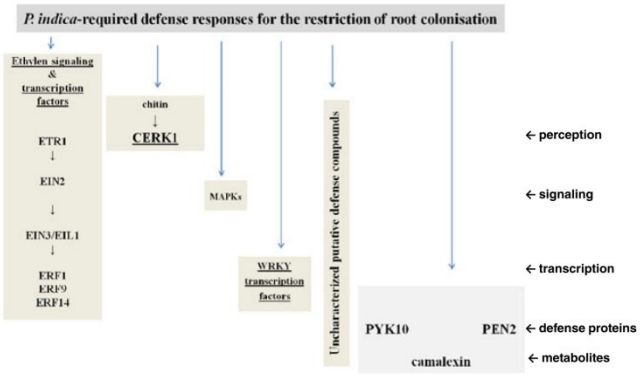

Fig. 3 Defense response components required for restricting Arabidopsis root colonization by *P. indica*. The components are involved in perception, signaling and transcription, or represent defense proteins or secondary metabolites

reductase5 (*dhar5*; SALK_029966C) T-DNA insertion lines (Vadassery et al. 2009c) were studied in the resistance response, the ISR response against *A. brassicae* was lost. MDAR and DHAR are two enzymes of the ascorbate-glutathione cycle that maintain ascorbate in its reduced state. *MDAR2* (At3g09940) and *DHAR5* (At1g19570) expression was upregulated in the roots and shoots of Arabidopsis seedlings co-cultivated with *P. indica* (Vadassery et al. 2009c). It appears that *P. indica* establishes a reduced atmosphere in the roots and leaves which contributes substantially to the ISR response against *A. brassicae* infections in leaves.

7 Novel compounds involved in *P. indica*/plant symbioses

Novel genes/proteins which are required for the restriction of root colonization were also identified. One of these proteins is At2g40000, called HSPRO [an ORTHOLOG OF SUGAR BEET Hs1(pro-1)]. The role of this protein in Arabidopsis is not clear, but recent studies with *Nicotiana attenuata* have shown that HSPRO controls early seedling growth during interaction with *P. indica* (Schuck et al. 2012). *HSPRO* expression was induced during herbivory, when leaves were inoculated with *Pseudomonas syringae* pv tomato DC3000 and roots with *P. indica*. Reduced *HSPRO* expression positively influenced early seedling growth during interaction with *P. indica*; fungus-colonized seedlings with reduced *HSPRO* expression increased their fresh biomass by 30 % compared to the wild type. Grafting experiments demonstrated that reduced *HSPRO* expression in roots was sufficient to induce differential growth promotion in both roots and shoots. This effect was accompanied by changes in the expression of 417 genes in colonized roots, most of which were metabolic genes. The lack of major differences in the metabolic profiles suggested that accelerated metabolic rates were involved. Therefore, HSPRO participates in a whole-plant change in growth physiology when seedlings interact with *P. indica* (Schuck et al. 2012). It would be interesting to see whether the Arabidopsis homolog has a similar function, and whether HSPRO couples growth and defense responses to the metabolic state of the plant.

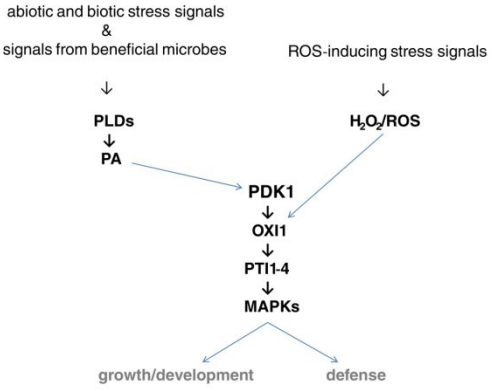

Fig. 4 A model describing the PDK1/OXI1 pathway and its potential involvement in balancing growth/development and defense responses

8 Balancing defense and growth: role of 3-PHOSPHOINOSITIDE-DEPENDENT-KINASE1 (PDK1) and OXIDATIVE-SIGNAL-INDUCIBLE1 (OXI1) in the symbiotic interaction

In natural environments plants either put their energy into growth and development or defense against enemies or pathogens. In a friendly environment, most of the energy is put into growth, and the synthesis of constitutive defense compounds ensures that the plants are protected against mild pathogen attacks. As soon as the plants are exposed to severe attacks by microbes, nematodes, herbivores, etc., the metabolism has to be readjusted or be reprogrammed to

activate induced defense responses. How does the plant balance defense and growth responses?

The roots have to monitor the microbial community in the rhizosphere continuously to establish an appropriate response and to integrate the incoming information from beneficial and pathogenic microbes. Thus, the roots have to identify whether an interacting microbe is a friend or a foe. Mycorrhizal fungi such as *Glomus intraradices* secrete symbiotic signals that are a mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs), which stimulate formation of arbuscular mycorrhizal fungi in plant species of diverse families (Fabaceae, Asteraceae and Umbelliferae) (Maillet et al. 2011). Studies on mycorrhiza have demonstrated that initially, the plant activates a mild defense response against the fungus, before a mutualistic interaction will be established and the microbe is accepted as a friend. Which kind of signals trigger this change is as yet unknown, but it has been proposed that the establishment of a mutualistic interaction starts with the exchange of nutrients between the two symbionts (Harrison 1999). Studies with the Arabidopsis/*P. indica* symbiosis suggest that the PDK1/OXI1 pathway plays a crucial role in this scenario (Camehl et al. 2011; Hirt et al. 2011).

An important second messenger in plant signaling is phosphatidic acid (PA) which can be synthesized either by phospholipase D (Li et al. 2009) or by a phospholipase C pathway which generates diacylglycerol that is phosphorylated to PA via diacylglycerol kinase (Arisz et al. 2009). Both lipases are activated in response to many biotic and abiotic stress signals (Li et al. 2009; Arisz et al. 2009). Although the beneficial fungus *P. indica* stimulates PA synthesis, this does not lead to defense gene activation, but the promotion of growth and plant performance (Camehl et al. 2011; Hirt et al. 2011). Therefore, the PA/PDK1/OXI1 pathway may integrate various external signals in plants to coordinate appropriate downstream responses, such as defense against pathogens and a mutualistic interaction with beneficial microbes. PA binds to PDK1 (Deak et al. 1999). In mammalian systems PDK1 is a master kinase, and more than 100.000 publications have shown that this kinase plays essential roles in cell growth, proliferation, survival, metabolism and apoptosis. Both mammalian and plant PDK1 phosphorylates and thus activates the cAMP-dependent protein kinase A/cGMP-dependent protein kinase G/protein kinase C (AGC) kinases in response to rises in the levels of signaling lipids (Bayascas 2010; Mora et al. 2004). In plants, PDK1 phosphorylates and thus activates the AGC kinase OXI1 in Arabidopsis (Anthony et al. 2004) and in rice (Matsui et al. 2010b) or Adi3 (AvrPto-dependent Pto-interacting protein 3) in tomato (Devarenne et al. 2006). In contrast to mammals, *pdk1* knock-out lines in Arabidopsis and rice are not lethal (Camehl et al. 2011) and OXI1 can still be activated in Arabidopsis PDK1-RNAi knock-down lines.

OXI1 can also be activated independently of PA/PDK1. Important stimuli for PA/PDK1-independent OXI1 activation are H_2O_2 and the pathogen-associated molecular pattern (PAMP) flagellin (Li et al. 2009). H_2O_2 accumulates in plants during pathogen attack, but not after co-cultivation with the beneficial fungus *P. indica*. Therefore, signals from pathogens and beneficial microbes come together at this pathway and it could integrate signals from different microbes in the environment. OXI1 was shown to be required for reactive oxygen species (ROS)-mediated responses in Arabidopsis such as root hair elongation and for disease resistance to biotrophic pathogens (Rentel et al. 2004; Petersen et al. 2009). The kinase activity of OXI1 itself was induced by H_2O_2, wounding, cellulase and various elicitor treatments mimicking pathogen attack (Anthony et al. 2006; Rentel et al. 2004). Furthermore, *oxi1* mutant plants are impaired in the activation of MPK3 and MPK6 in response to cellular injury and oxidative stress (Rentel et al. 2004). OXI1 is an upstream regulator of stress-responsive PTI1 (Anthony et al. 2006; Forzani et al. 2011; Matsui et al. 2010a) and MPKs although the mechanism is still unclear. PTI1 proteins are Ser/Thr protein kinases that share sequence identity to tomato PTI1 (Pto-interacting 1). In tomato, PTI1 is phosphorylated by the Ser/Thr kinase Pto conferring resistance to *P. syringae* expressing the effector AvrPto and positively regulates the cell death response triggered by Pto (Martin et al. 1993; Zhou et al. 1995). In contrast, rice PTI1a inhibits disease resistance and cell death and is negatively regulated by OsPDK1-OsOXI1 signaling cascade in response to ROS and PAMP treatments (Matsui et al. 2010a; Takahashi et al. 2007).

OXI1 is the responsible gene for the growth phenotype induced by *P. indica* (Camehl et al. 2011). OXI1 can be activated by H_2O_2 (and therefore stress signals from pathogens) and by PA/PDK1 (activated by biotic and abiotic stress signals and signals from the beneficial fungus *P. indica*). Root colonization by the fungus stimulates PA synthesis in Arabidopsis plants. These results suggest that *P. indica* stimulates growth by PA-mediated activation of PDK1 which subsequently activates OXI1. ROS production is not stimulated and is even inhibited by the beneficial fungus and thus does not play a role in activating OXI1 (Camehl et al. 2011).

In conclusion, we propose that the PDK1-OXI1 signaling pathway (either directly or by activating downstream components) plays a crucial role in integrating signals from pathogenic and beneficial fungi to induce either defense gene activation or the promotion of growth and development.

9 Conclusions

The data summarized here demonstrate that establishing or maintaining a beneficial symbiotic interaction between *P. indica* and Arabidopsis strongly depend on the defense repertoire of the host. A main function of the host defense is to control hyphal growth in the roots, and consequently genetic

inactivation of specific defense compounds results in uncontrolled fungal growth. It appears that this control mechanism is not associated with a particular defense process, but that the mixture of the different defense strategies available for a particular plant or species is probably crucial for a fined-tuned communication between the beneficial symbionts. Consistent with this observation, we identified genes and proteins which participate in the activation of defense processes at different levels (perception of envionmental signals, plant signal transduction, transcription, defense proteins and compounds; Fig. 3). Interestingly, impairments in a particular defense process often lead to a compensatory upregulation of other, unrelated defense processes to restrict fungal growth. Overall, these defense processes are only mildly activated in roots colonized by the beneficial fungus *P. indica*, and it is conceivable that a strong defense response from the host would result in less root colonisation and consequently a disturbed balance in the symbiosis. Finally, the host has to decide whether it puts its energy and resources into growth or defense. This requires a highly sophisticated sensing of the microbial environment. Any wrong decision has severe consequences for the fitness and survival chance of the plant. Consequently, there must be a crosstalk between signaling events leading to defense and those activating growth and development. The AGC kinases fufill the requirements to integrate signals which are beneficial and non-beneficial for the plant, and have the capability to initiate processes leading to a balanced response between growth, development, defense and cell death (cf. Garcia et al. 2012).

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4.6 Manuscript VI

Vahabi K, Johnson JM, Drzewiecki C, Oelmüller R (2011) Fungal staining tools to study the interaction between the beneficial endophyte *Piriformospora indica* with *Arabidopsis thaliana* roots. *Journal of Endocytobiosis and Cell Research* 21:77-88.

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Journal of Endocytobiosis and Cell Research

Technical note:

Fungal staining tools to study the interaction between the beneficial endophyte *Piriformospora indica* with *Arabidopsis thaliana* roots

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Abstract

Piriformospora indica, a primitive endophytic fungus of Sebacinales, colonizes the roots of all plant species investigated so far and promotes plant growth. It also increases seed production, uptake of nutrients and resistance to biotic and abiotic stress. Several studies have demonstrated the degree of root colonization is crucial for the benefits of both symbionts. Furthermore, since the fungus grows inter- and intra-celullarly, efficient tools are required for the detection of fungal hyphae and spores in and around the roots. Here we describe different staining methods to study the histology of *P. indica-Arabidopsis thaliana* interaction.

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Introduction

Piriformospora indica is an endophytic fungus of Sebacinales that colonizes the roots of many plant species including forestry, agricultural, horticultural and medicinal species (Barazani et al. 2005; Glen et al. 2002; Kaldorf et al. 2005; Oelmüller et al. 2005; Peškan-Berghöfer et al. 2004; Pham et al. 2004; Sahay and Varma 1999; Selosse et al. 2002a, b; Shahollari et al. 2005, 2007; Sherameti et al. 2005, 2008a, b; Urban et al. 2003; Varma et al. 1999, 2001; Waller et al. 2005; Weiss et al. 2004), as well as the model plant *Arabidopsis thaliana* (cf. Peškan-Berghöfer et al. 2004; Oelmüller et al. 2005; Shahollari et al. 2005; Sherameti et al. 2008a; Camehl et al. 2011). For all plant species tested so far, *P. indica* showed to have the potential to promote growth and biomass production indicating that the symbiosis is beneficial for the hosts and has the potential for agricultural and biotechnological applications. We use Arabidopsis as host to understand the molecular basis of the beneficial interaction between the symbionts.

P. indica hyphae can be detected on the surface of Arabidopsis root, in the outer cell layers, and within the root cells, using optical microscopy (Peškan-Berghöfer et al. 2004). Several studies have demonstrated that the degree of root colonization is critical for establishing benefits for the plants. For the wild-type, root colonization and association of fungal hyphae results in the promotion of growth and seed yield, as well as tolerance against biotic and abiotic stress (Varma et al. 1999; Verma et al. 1998; Oelmüller et al. 2009; Oelmüller et al. 2004; Rai et al. 2004; Sherameti et al. 2008a, b; Varma et al. 2001; Waller et al. 2005), whereas overcolonization of hyphal growth can result in the loss of the benefits, and may even lead to a shift from a mutualistic to a antagonistic interaction (Johnson and Oelmüller 2009; Camehl et al. 2010a, b). Furthermore, it has been proposed that the beneficial interaction of *P. indica* with barley roots requires cell death programs (Deshmukh et al. 2006; Schäfer et al. 2007). Fungal colonization increases with root tissue maturation. The root tip meristem of barley showed no colonization, and the elongation zone showed mainly intercellular colonization. In contrast, the differentiation zone was heavily infested by inter- and intracellular hyphae and intracellular chlamydospores. The majority of hyphae were present in dead rhizodermal and cortical cells that became completely filled with chlamydospores. In some cases, hyphae penetrated cells and built a meshwork around plasmolyzed protoplasts, suggesting that the fungus either actively kills cells or senses cells undergoing endogenous programmed cell death. Further studies with the barley BAX inhibitor-1, a gene capable of inhibiting plant cell death, demonstrated that *P. indica* requires host cell death for proliferation in differentiated barley roots (Deshmukh et al. 2006; Schäfer et al. 2007). Recently, this study was extended to Arabidopsis (Jacobs et al. 2011). By employing transmission electron microscopy and epifluorescence microscopy along with reporter and mutant plants, the authors observed a biotrophic colonization of Arabidopsis roots by a fungus. This biotrophic stage is followed by a cell death-associated colonization phase. The genetic and molecular analyses also demonstrated the efficiency of the root innate immune system to halt microbial colonization and indicated that mutualistic colonization success is intimately dependent on efficient immune suppression strategies (Jacobs et al. 2011).

All these observations clearly demonstrate that powerful staining techniques are required to visualize hyphal growth within the roots.

Until now, the growth and morphology of *P. indica* hyphae and spores was studied by light microscopy, epifluorescense, scanning-electron (Sun et al. 2010) or confocal microscopy (Stein et al. 2008; Waller et al. 2005). However,

some of the light microscopical techniques were time-consuming and harsh (Verma et al. 1998). Recently, GFP-transformed *P. indica* strains have been reported (Zuccaro et al. 2009), which might be a powerful tool in future studies.

We use *Arabidopsis thaliana* as a host for *P. indica* and employed various simple methods for the visualization of the spores and hyphae within and around the root. This includes live cell microscopy, different staining methods and epifluorescense microscopy. The objectives of this study were to develop fast and simple methods to describe the colonization under different growth and cultivation conditions, and to obtain staining protocols that allow study of the fungal growth to some extend in and around the Arabidopsis roots.

Results and Discussion

The interaction of *P.* indica with A. thaliana roots was studied on different media including PNM (Plant Nutrient Medium) and MS (Murashige and Skoog-Salt) by light and fluorescence microscopy without chemical treatment (Figures 1-7). With all methods, the mycelium is nicely visible in *A. thaliana* roots by optical microscope. Using different

Figure 1: *Arabidopsis thaliana* roots, and hyphae of *P. indica* under the light microscope, magnification 400x. Life microscopy. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

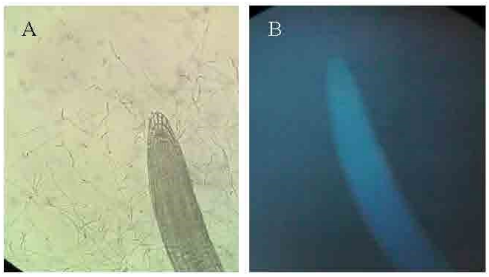

Figure 2: *Arabidopsis thaliana* roots, and hyphae of *P. indica* under the light microscope (A) and fluorescent channel at 450-490 nm (B), magnification 100 times. Life microscopy. 15 days after interaction of *P. indica* with *A. thaliana* on PNM .medium.

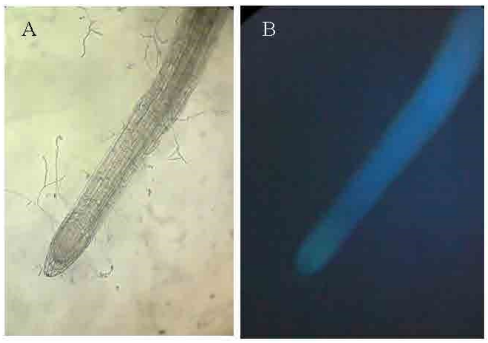

Figure 3: *Arabidopsis thaliana* roots, and hyphae of *P. indica* under the light microscope (A) and fluorescent channel 450-490 nm (B), magnification 100x. Life microscopy. 15 days after co-cultivation of *P. indica* with *A. thaliana* on MS-S medium.

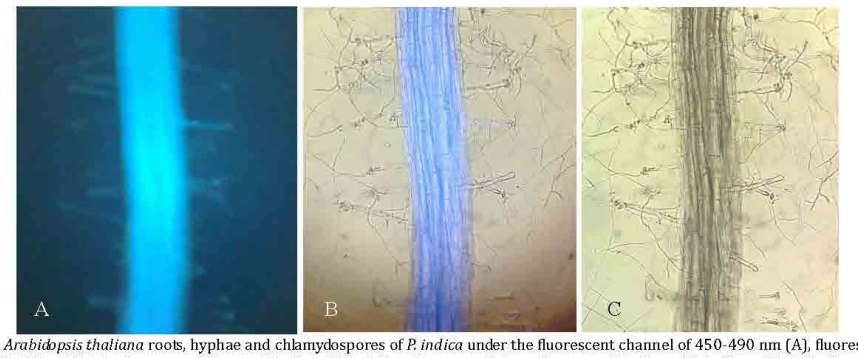

Figure 4: *Arabidopsis thaliana* roots, hyphae and chlamydospores of *P. indica* under the fluorescent channel of 450-490 nm (A), fluorescent and visible light (B) and light microscopy (C), magnification 100x. Life microscopy. 7 days after co-cultivation of *P. indica* with *A. thaliana* on MS-S medium.

magnifications (4x, 10x, 20x and 40x) and different wavelengths (visible and fluorescent channels) better resolution and discrimination of fungal and plant tissues could be achieved. Fluorescence microscopy is more sensitive compared to light microscopy. Additionally, different layers of the cell tissue can be scanned using fluorescence microscopy.

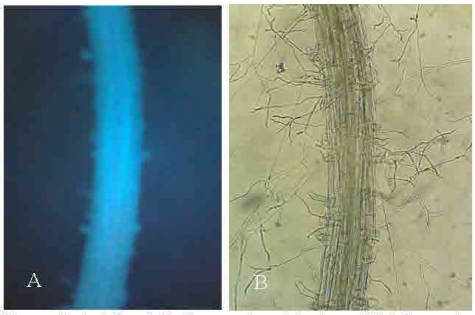

Figure 5: *Arabidopsis thaliana* root, and hyphae of *P. indica* under the fluorescent channel at 450-490 nm (A) and under the light microscope (B), magnification 100x. Live microscopy. 15 days after co-cultivation of *P. indica* with *A. thaliana* on MS-S medium.

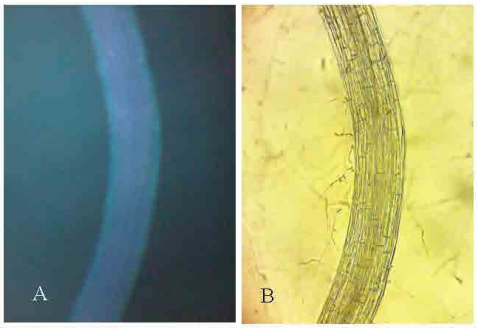

Figure 6: *Arabidopsis thaliana* root, and hyphae of *P. indica* under the fluorescent channel at 450-490 nm (A) and under light microscopy (B), magnification 100x. Live microscopy. 7 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

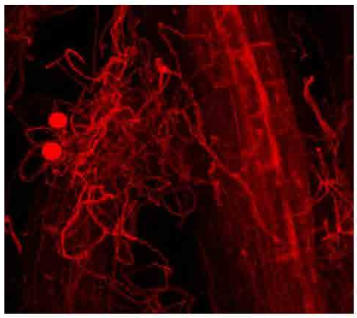

Figure 7: *Arabidopsis thaliana* root, and hyphae of *P. indica* under the fluorescence channel at 488 nm. Life microscopy. magnification 400x. 4 weeks after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

Fluorescence microscopy

Optimal results were obtained with fuchsin acid and fluorescence microscopy using different wavelengths (Figures 8-10). The method is fast and allows visualization of *P. indica* mycelia and chlamydospores in the root environment. For detection and counting of chlamydospores, we found that staining with erythrosine and safranin using the fluorescence channel at 470 and 450-490 nm resulted in contrast between spore and roots (Figure 11A and B). With this method the mycelia are not visible and the spores can be counted without light interference. Staining with fuchsin acid/cotton blue gave comparable results. With this method, all stained parts of the roots and mycelia can be seen with visible light, but at 470 nm, only chlamydospores are visible (Figure 12).

Chemical staining

The optimal time for staining with Trypan blue (0.0001 mg/ml) is 1 minute, since longer incubations also stains root tissue. This method gave best results when only the mycelium should be visualized. It shows the interaction with root environment and allows the analysis of the mycelium networks in and around the roots. Furthermore, the strong contrast between the colored mycelia and the background is suitable for the analysis of root colonization with appropriate software (Figures 13 and 14).

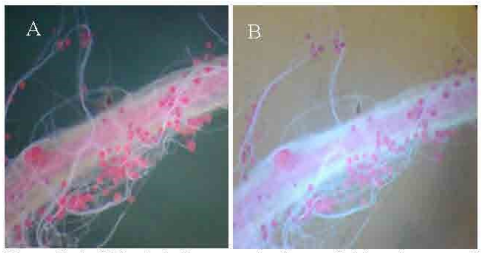

Figure 8: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 450-490 nm (A) and at 365 nm (B); magnification 100x. Fuchsin acid staining method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

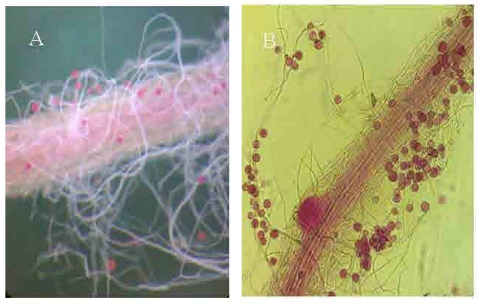

Figure 9: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 450-490 nm (A, magnification 200x) and under light microscopy (B, magnification 100x). Fuchsin acid staining method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

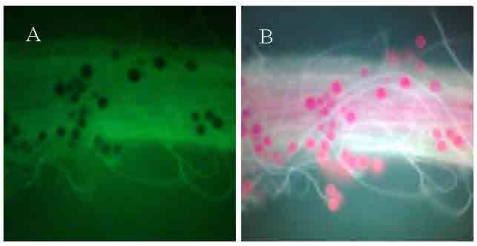

Figure 10: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 470 nm (A) and 450-490 nm (B), magnification 200x. Fuchsin acid staining method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

Figure 11: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 470 nm (A), 450-490 nm (B) and under the light microscope (C), magnification 200x. Erythrosine/safranin method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

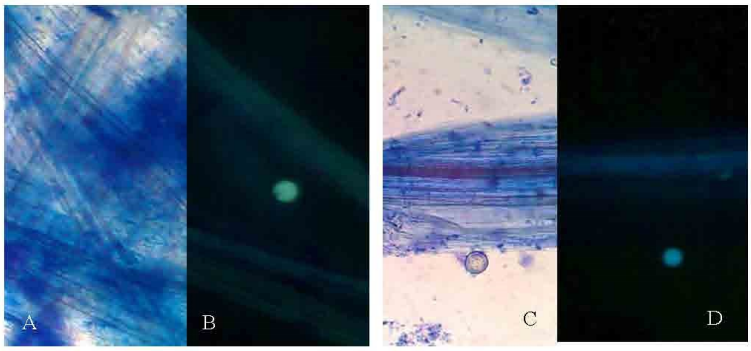

Figure 12: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope (A and C) and fluorescent channel at 470 nm (B and D), magnification 400x. Fuchsin acid / cotton blue method 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

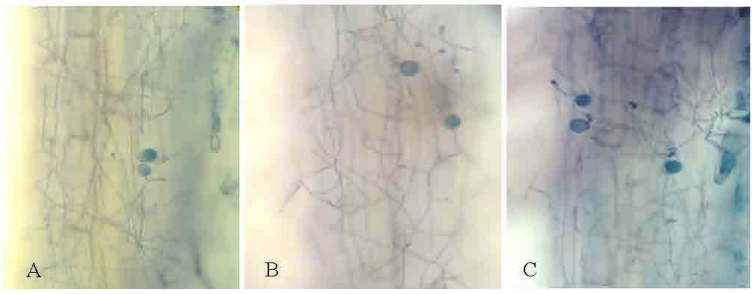

Figure 13: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* light microscopy, magnification 200x. Trypan blue (0.0001 mg/ml) method (A, B, 1 min staining; C, 5 min staining). 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

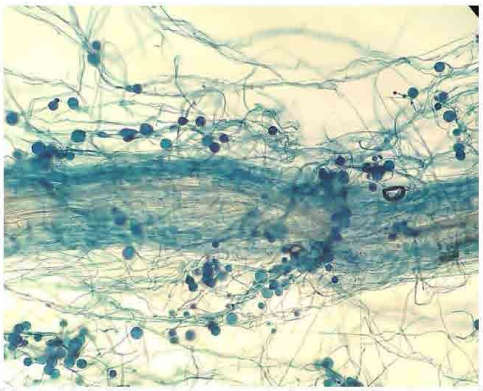

Figure 14: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope, magnification 200x. Trypan blue (0.01 mg/ml) method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

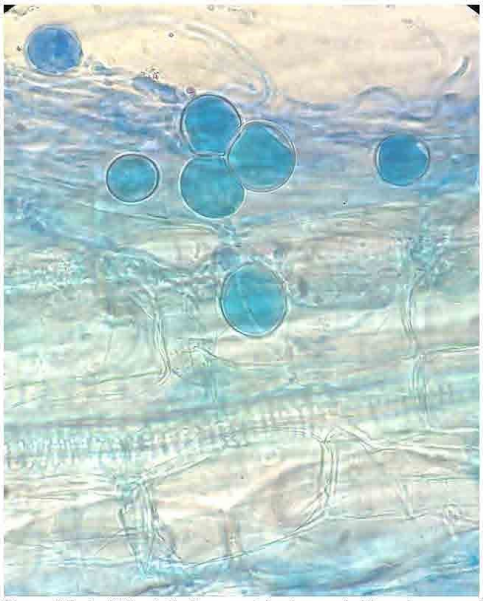

Figure 15: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under light microscope, magnification 1000x. Erythrosine/aniline blue method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

By staining spores in the root structure we obtained optimal results with the erythrosine and aniline blue stain, since it allows visualization of both root tissue and cells (Figure 15). Other methods used were staining with janus green/methyl green/natural red, methyl green/aniline blue, naphtol blue black, fuchsin acid/trypan blue, cotton blue (Figures 14-23).

In conclusion, different staining methods allow the identification of the fungal mycelium and spores in the root environment, and partly the quantification of root colonization. We used combinations of these staining methods to identify mutants which are impaired in the *P. indica*/Arabidopsis interaction and to identify plant mutants which fail to host the fungus. Besides basic research to understand the molecular mechanisms underlying root colonization and propagation of the mycelium, the comparative analysis of the staining methods may also help to analyze colonization of the roots from agriculturally important plant species.

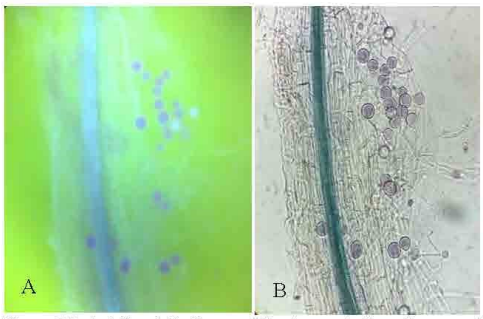

Figure 16: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 450-490 nm (A) and under the light microscope (B), magnification 200x. Janus green/methyl green/natural red staining method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

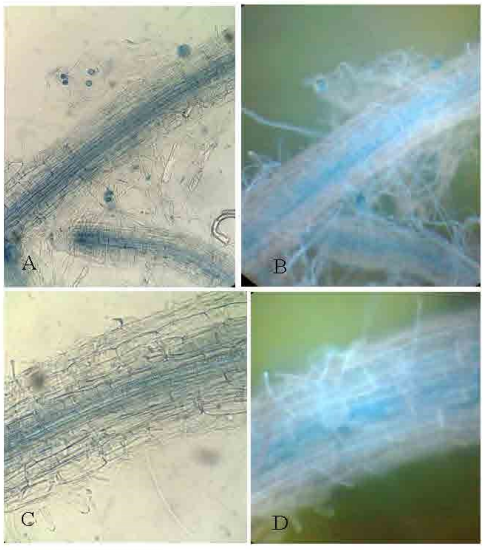

Figure 17: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope (A and C) and under the fluorescent channel at 450-490 nm (B and D), magnification 200x (A and B), 400x (C and D). Methyl green/aniline blue staining method. 15 days after interaction of *P. indica* with *A. thaliana* on PNM medium.

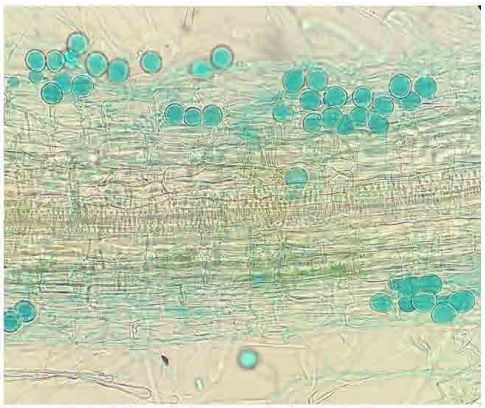

Figure 18: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope, magnification 400x. Naphtol blue black method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

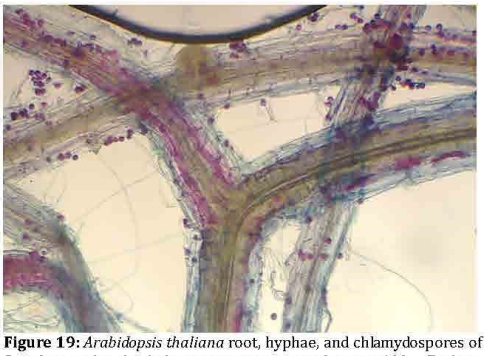

Figure 19: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope, magnification 100x. Fuchsin acid/trypan blue method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

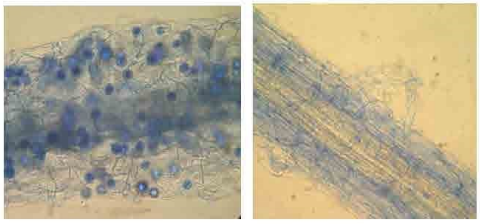

Figure 20: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope, magnification 400x. Cotton blue staining method. 2 weeks after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

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The Methods

Growth conditions for *Piriformospora indica* and Arabidopsis

Piriformospora indica was cultured as described previously (Verma et al. 1998; Peškan-Berghöfer et al. 2004) in Petri dishes on a modified Kaefer's medium (KM). The plates were kept at room condition for 2 weeks. For co-cultivation experiments with Arabidopsis, *P. indica* spore solutions (1×10^6 spore/ml) were prepared from the fungus grown on KM plates. Root inoculation by *P. indica* was preformed by adding 5 µl of spore solution (1×10^6 spore/ml) to each seed on different media including MS (Murashige and Skoog) and PNM.

Life microscopy

Step 1 Plant 10 seeds of *A. thaliana* and 5 µl of *P. indica* spores ($1x10^6$ s/ml) on a PNM or MS plate
Step 2 Keep at 4°C for 2 days before transfer to light (24 hour light and 23°C) for 10 days
Step 3 Observe the roots by light or fluorescent microscopy directly in the plates.

Staining with fuchsin acid

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into fuchsin acid solution for 5 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with erythrosine/safranin

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into erythrosine solution for 1 min.
Step 3 Stain with safranin solution for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with fuchsin acid and cotton blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into fuchsin acid solution for 2 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Stain with cotton blue solution for 1 min.
Step 5 Washing the samples in distilled water for 1 min.
Step 6 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 7 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with janus green/methyl green/natural red

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into janus green and methyl green mixture solution (1:1) for 2 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Stain with natural red solution for 1 min.
Step 5 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 6 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with erythrosine and aniline blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into erythrosine solution for 2 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Stain with aniline blue solution for 1 min.
Step 5 Washing the samples in distilled water for 1 min.
Step 6 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 7 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with trypan blue (0.0001mg/ml)

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample into trypan blue solution for 1 or 5 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths

Staining with trypan blue (0.01 mg/ml)

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample into trypan blue solution for 1 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths

Staining with naphtol blue black

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample in naphtol blue black solution for 1 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with fuchsin acid and trypan blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample in fuchsin acid solution for 10 min.
Step 3 Washing the samples in distilled water for 1 min.
Step 4 Stain with trypan blue (0.0001gm/ml) solution for 3 min.
Step 5 Washing the samples in distilled water for 1 min.
Step 6 Adding 50 µl GL solution on glass slide and cover with glass cover.
Step 7 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with methyl green and aniline blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample in methyl green (0.05%) solution for 10 seconds.
Step 3 Stain with aniline blue solution for 1 min.
Step 4 Washing the samples in distilled water for 1 min.
Step 5 Adding 50 µl GL solution on glass slide and cover with glass cover.
Step 6 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with cotton blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample in cotton blue solution for 30 seconds.
Step 3 Put on glass slide and cover with glass cover.
Step 4 Observe the samples by light and fluorescent microscopy using different wavelengths.

Media and Solutions

MS Medium

MS-Salt (=Murashige and Skoog-Salt)	4.4 g
Gel-Rite	3 g
MES	0.5 g
Saccharose	13.7 g
H_2O	up to 1 litter

pH 5.7 – 5.8

PNM Medium

KNO_3	5 mM
$MgSO_4$ 1 M	2 mM
$Ca(NO_3)_2$ 1 M	2 mM
$FeSO_4$	0.01 mM
H_3BO_3	70 mM
$MnCl_2$	14 mM
$CuSO_4$	0.5 mM
$ZnSO_4$	1 mM
Na_2MoO_4	0.2 mM
$CoCl_2$	0.01 mM
Gel-Rite	3 g
KH_2PO_4 1 M	2.5 ml
H_2O	up to 1 liter

pH 5.6

Kaefer's medium

$NaNO_3$	7.0 mM
KCl	7.0 mM
$MgSO_4$	2.1 mM
KH_2PO_4	9.2 mM
EDTA	0.02 mM
$ZnSO_4$	0.77 mM
H_3BO_4	0.18 mM
$MnSO_4$	0.02 mM
$CoCl_2$	0.007 mM
$CuSO_4$	0.0065 mM
$FeSO_4$	0.02 mM
ammonium molybdate	0.001 mM
thiamine	0.003 mM
gylcine	0.005 mM
nicotinic acid	0.002 mM
pyridoxine	0.0004 mM
glucose	110 mM
peptone	2 g/l
yeast extract	1 g/l
casein hydrolysate	1 g/l
agar	10 g/l
H_2O	up to 1 liter

pH 6.5

Glycerol lactic acid solution (GL)	
glycerol	10 ml
lactic acid	10 ml
distilled water	10 ml

Trypan blue (0.0001mg/ml) solution	
GL solution	1 ml
trypan blue	0.0001 g

Trypan blue 0.01mg/ml solution	
GL solution	1 ml
trypan blue	0.01 g

Fuchsin acid solution	
GL solution	1 ml
fuchsin acid	0.001 g

Erythrosine solution	
GL solution	1 ml
erythrosine	0.01 g

Aniline blue solution	
GL solution	1 ml
aniline blue	0.005 g

Naphtol blue black solution	
GL solution	1 ml
naphtol blue black	0.0001 g

Safranin solution	
LG solution	1 ml
safranin	0.0001 g

Janus green solution	
GL solution	1 ml
janus green	0.0001 g

Methyl green solution	
GL solution	1 ml
methyl green	0.0001 g

Natural red solution	
GL solution	1 ml
natural red	0.0001 g

Cotton blue solution (Lactophenol cotton blue solution)	
glycerol	50 g
phenol	25 g
L(+)-lactic acid	25 g
cotton blue	50 mg

4.7 Manuscript VII

Vahabi K, Meischner D, Oelmüller R (2014) Interaction of Arabidopsis and *Piriformospora indica* in a hydroponic system. *Journal of Endocytobiosis and Cell Research* 25: 63-65.

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Journal of Endocytobiosis and Cell Research

Interaction of Arabidopsis and *Piriformospora indica* in a hydroponic system

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In this brief method paper, we describe the co-cultivation of Arabidopsis seedlings with the beneficial root-colonizing fungus *Piriformospora indica* in a hydroponic medium and discuss the advantages of this system to study the interaction of the two symbionts and the identification of chemical mediators which participate in their cross-talk.

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Introduction

Interaction between the endophytic fungus *Piriformospora indica* and Arabidopsis roots can be beneficial for both partners resulting in plant growth promotion and/or enhanced stress tolerance (Varma et al. 1999; Shahollari et al. 2007; Sherameti et al. 2005; Camehl et al. 2010; Nongbri et al. 2012; Zuccaro et al. 2009). However, the mode of this interaction on the molecular level is only poorly understood and appears to depend strongly on the co-cultivation conditions. Many open questions exist yet, which require standardized and reproducible cultivation conditions.

Most of the existing model systems are based on the interaction on solid substrates like defined nutrient media including agar or gelrite (Peškan-Berghöfer et al. 2004), vermiculite (Camehl et al. 2011), or clay beads (Schedel et al. 2012). All of them have different advantages and disadvantages, depending on the respective need of the experimental approach. For instance, investigation of the interaction on agar media with defined nutrient concentrations allows short time interaction studies with seedlings, vermiculite is optimal for long term interaction studies with adult plants and in particular for the analysis of the aerial parts of the plants, while clay beads can be used for long term studies of both shoots and roots, since removal of undamaged roots is relatively easy. All of these experimental approaches enable cultivation under defined conditions such as light, temperature, nutrient availability and sterility. However, various parameters can be limiting in these interaction systems, which cannot easily be manipulated, e.g. by applying additional components or substrates, and the experimental conditions are quite different from those found in nature. Often, these plant-microbe interaction systems represent stress for the symbionts, e.g. because of non-homogeneous distribution of the compounds, or the presence of compounds which are normally not available or not available in such amounts in nature (e.g. sugars). In addition, the experimental set-up is time-consuming, and restricted by the analysis of root development under vital conditions.

One solution for solving these problems might be the use of aqua culture and hydroponic systems. Hydroponic cultivation allows plants to grow in a solution with different nutrients and oxygen supply can be provided by an aeration system. In aqua cultures, plants grow in liquid media without aeration.

Application of hydroponic systems was described for analysis of plant defense (Fakhro et al. 2007; Shibuya and Minami 2001), changes in H_2O_2, phospholipase D and phosphatidic acid levels (Yamaguchi et al. 2004), different phosphate concentrations (Malá et al. 2010), salt stress (Maaroufi-Dguimi et al. 2011) and heavy metal bioremediation (Tu and Ma 2004; Wang et al. 2002; Mleczek et al. 2010) with different plant species.

Compared to model experiments on solid medium, beneficial interaction studies can be studied in hydroponic culture systems as well. With these experimental set-ups, the above ground plant tissue is exposed to air whereas the root system is arranged in liquid medium with defined nutrient composition. In order to prevent oxygen deficiency (if required) on the roots, the liquid media need to be aerated by a pump. Effects of the interaction on the plant, such as growth promotion or stress, can also be easily observed, measured and quantified in these systems. An advantage is the easy and controlled apply of specific compounds like elicitors, hormones, radioisotopes or chemicals. Furthermore, these arrangements are time saving, provide better homogeneity in the liquid media, are physical and mechanical stress-free (especially in aqua culture, because there is no aeration and disturbing factors for root growth and development). The roots of the seedlings or plants can grow in a pathogen- and pest-free environment or other

disturbing agents. Also secretome studies of the plant roots with and without the microbes or of the microbes alone are feasible. Other advantages of these cultivation conditions are that the analysis of intact roots without any damage is possible, because they do not need to be removed from solid agar or cleaned from other substrates. Furthermore, the seedlings with intact roots can be easily transferred to other media, or additional substances which are under investigation can be added without harm to the roots.

Figure1: Germinated *A. thaliana* seeds inoculated with *P. indica* chlamydospores in microtubes. (**A-C**) Arabidopsis roots colonized by *P. indica* 3 weeks after transfer to the sterile aqua culture condition. (**D** and **E**) Roots and the aerial parts of *A. thaliana* seedlings co-cultivated with *P. indica* 4 weeks after transfer to the non-sterile aqua culture (**F**) *A. thaliana* control seedlings and those co-cultivated with *P. indica* 4 weeks after transfer to the non-sterile aqua culture.

The aim of this technical note is to describe a convenient model system which allows to analyze the interaction between plants and fungi, such as the beneficial root-colonizing fungus *P. indica* in aqua and hydroponic cultures. Our results show that the plants grow healthy, allow morphological studies in particular of the root architecture, handling of the shoots and roots is easy and translocation of plant to different media do not cause mechanical and physical stresses for the seedlings or plants (Figures 1 and 2). Microscopic studies show that root colonization by *P. indica* mycelium (Figure 3E-H) occurs when spores are added to the medium. Furthermore chlamydospores of *P. indica* were detected in the older parts of the roots (Figure 3E-H).

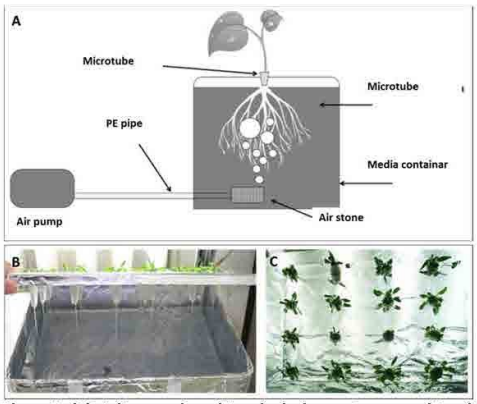

Figure2: (A) A diagram describing the hydroponic system. (**B** and **C**) Arabidopsis seedlings colonized by *P. indica* two weeks after transfer to the hydroponic system.

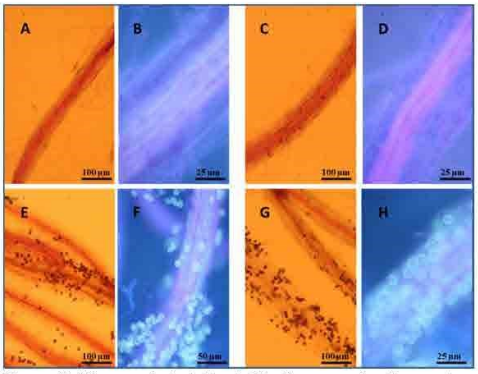

Figure 3: Microscopical study of *P. indica* spore development on Arabidopsis roots in aqua culture (**A, B, E** and **F**) and hydroponic culture (**C, D, E** and **F**). **A-D**: Control roots not inoculated with fungal spores. **E-F**: Roots inoculated with *P. indica* spores. Arabidopsis roots without *P. indica* (left), Arabidopsis root hair in the presence of *P. indica* (right). The pictures show chlamydospores of *P. indica* under visible light (**A, C, E** and **G**) and under the fluorescent channel at 450-490 nm (**B, D, F** and **H**).

Material and Methods

Collection of *P. indica* spores

P. indica was cultivated on solified Käfer medium (Käfer 1977) at 25 °C in the dark. After 4 weeks, deionized water was added to the fungal culture grown on solid medium and spores were collected with a scalpel. The material was subsequently washed three times with sterile water and collected by centrifugation (2000 rpm for 1 min). The spore concentration was determined by counting and adjusted to 10^6 spores/ml distilled water.

Co-cultivation of Arabidopsis and *P. indica*

For co-cultivation of Arabidopsis with *P. indica*, sterilized 1.5 ml Eppendorf tubes were filled with 1.5 ml PNM medi-

um [5 mM KNO_3, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.01 µM $FeSO_4$, 70 µM H_3BO_3, 14 µM $MnCl_2$, 0.5 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.2 µM Na_2MoO_4, 0.01 µM $CoCl_2$, 3 g gelrite, and the pH was adjusted to 5.6]. The Eppendorf microtube lids were removed and sterilized in autoclavable boxes. For the inoculation of Arabidopsis seeds with the *P. indica* spores, 2 µl of the spore solution were placed to each seed (Figure 1). Uninoculated control seeds and inoculated seeds were transferred to 4 °C for 48 h. After cold treatment, the microtube were incubated at 23 °C and kept under long day conditions (light intensity: 65 µmol m^{-2} sec^{-1} from the top) for 10 days, before transferring to the aqua culture or hydroponic systems (Figure 1C).

Transfer of seedling to aqua culture

The aqua culture tank is a 300 ml container filled with liquid PNM which was with aluminum foil. It contained holes which allowed the stable insertion of the microtubes. Before transferring the plants to the tubes, the tips of the microtubes were cut off to allow the roots to grow into the liquid medium. The microtubes were put into the holes of the aluminum foil, and the container was covered with its lid (Figure 1). Experiments were carried out in long day light condition (16 h light/8 h dark, 65 µmol m^{-2} sec^{-1} from the side). After 2 weeks representative pieces of roots were stained with trypan blue and analyzed under the microscope for root colonization (Vahabi et al. 2011). For sterile co-cultivation conditions 300 ml-big Magenta boxes were used instead bigger plastid boxes (Figure 1D-E).

Transferring of seedling to hydroponic system

The hydroponic system was composed of a 5 l container filled with liquid PNM medium, a simple aquarium pump for aeration, and a pneumatic control system providing aeration of the medium by the pump. The arrangement is similar to that outlined above. The aeration system with sterile polyethylene pipes was positioned at the container bottom and the air was dispersed in the liquid media by an aeration stone (Figure 2). Experiments were performed under long day conditions (16 h light/8 h dark, 65 µmol m^{-2} sec^{-1} from the top). After 2 weeks representative pieces of the roots were stained with trypan blue and analyzed for colonization under a microscope as described above and in Vahabi et al. (2011). Plants were harvested at different time points for estimation of shoot and root biomass.

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4.8 Manuscript VIII

Vahabi K, Sun C, Govindaswamy J, Falkenberg D, Venus T, Oelmüller R (2015) Stomata staining in Arabidopsis. *Journal of Endocytobiosis and Cell Research* 26: 21-24.

Journal of Endocytobiosis and Cell Research (2015) 21-24 | International Society of Endocytobiology
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Journal of Endocytobiosis and Cell Research

Stomata staining in Arabidopsis

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Stomata on plant epidermis are crucial for gas exchange and water evaporation. The stomatal guard cells are reported to be involved in plant innate immunity response. Here we provide different staining methods which could help to investigate the stomata under natural conditions and after pathogen attack.

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Keywords: stomata, staining, *Alternaria brassicae*, *Arabidopsis thaliana*

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Introduction

Stomata are micrometric openings in the leaf epidermis which are responsible for gas exchange and controlling water evaporation during transpiration (Ou et al. 2014) and are good gates to let pathogenic bacteria and fungi penetrate inside the plant leaves. Stomata derive from incomplete cytokinesis mother guard cells (Budke et al. 2012) which result in formation of two epidermal kidney shape cells surrounding a micrometric aperture (Sachs 1991). Guard cells control the size of apertures by perception and response to a wide range of biotic and abiotic stresses (Hetherington and Woodward 2003; Ruszala et al. 2011; Chater et al. 2011; Bauer et al. 2013; Kim et al. 2010; Schroeder et al. 2001; Shimazaki et al. 2007; Xie et al. 2006). They play crucial roles as first plant obstacle and show rapid closing reaction after plant pathogen attack (Gudesblat et al. 2009; Melotto et al. 2006; Montillet et al. 2013).

Stomata function and structure have been previously studied by using light and electron microscopy (Garner and Paolillo 1973; Sack and Paolillo 1985). It is shown that these tiny surface pores play an important role in the homoiohydry (Cowan 1977; Raven 2002; Wong et al. 1979; Edwards et al. 1998; Franks and Beerling 2009; Berry et al. 2010; Haworth et al. 2011; Chater et al. 2013; Vialet-Chabrand et al. 2013; Raven and Edwards 2014). Their importance as a key player in controlling CO_2 and water exchange with the air is a big attraction and very important for the agriculture and horticulture (Jones 2004).

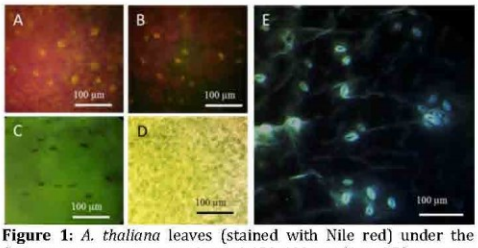

Figure 1: *A. thaliana* leaves (stained with Nile red) under the fluorescent microscope at channel 450-490 nm (A and B); staining with Aniline blue (C); without stain under visible light (D); stained with Methyl green under fluorescent channel 450-490 nm (E).

In this study we used different dyes (Table 1) for differential stomata staining and visualized them by light (Figures 1D, 3C) and fluorescent microscopy (Figures 1, 2, 3). Stomata staining after pathogenic fungal attack (*Alternaria brassicae*; Figures 4A, B, C), penetration through the aperture (Figures 3C, D, E and 4D, E, F) and colonization of guard cells (Figures 4G, H, I) by Alternaria mycelium have been studied.

Table 1: Staining solutions

Glycerol lactic acid solution (GL)	
Glycerol	10 ml
Lactic acid	10 ml
Tween 20	100 µl
Distilled water	10 ml
Calcofluor-white	
GL solution	1 ml
Calcofluor-white	0.0001 g
Fuchsin acid solution	
GL solution	1 ml
Fuchsin acid	0.001 g
Aniline blue solution	
GL solution	1 ml
Aniline blue	0.005 g
Methyl green solution	
GL solution	1 ml
Methyl green	0.0001 g
Nile red solution	
GL solution	1 ml
Nile red	0.0001 g

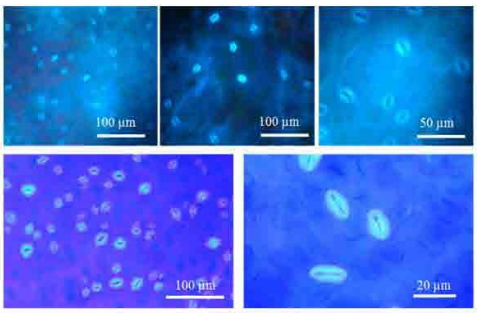

Figure 2: Different fluorescent microscopical views at channel 365 nm of *A. thaliana* leaves stained with Calcoflure.

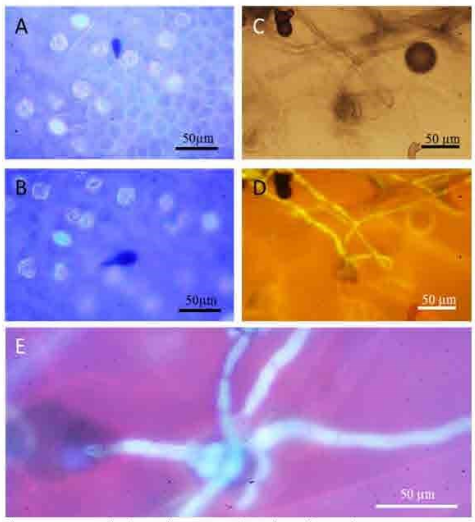

Figure 3: *A. thaliana* leaves infected with *A. brassicae* spores, stained with Calcoflour and observed under fluorescent microscope at channel 365 nm (A and B); Fuchsin acid staining of *A. brassicae* mycelium penetrating a stomata under visible light (C); fluorescent microscopy at channels 450-490 nm (D) and 365 nm (E).

Material and methods

Growth conditions of *Arabidopsis thaliana* and *Alternaria brassicae*

A. thaliana WT (ecotype Columbia-0) seeds were surface-sterilized and placed on Petri dishes containing MS nutrient medium (Murashige and Skoog 1962). After cold treatment at 4°C for 48 h, plates were incubated for 10 days at 22°C under long day condition (65 µmol m^{-2} sec^{-1}). Arabidopsis leaves were inoculated with 2 µl of *A. brasicae* spore solution and kept for one week at 22°C under long day condition (65 µmol m^{-2} sec^{-1}). *A. brassicae* was cultured for three weeks at 22-24°C on PDA and the collected spores were washed 3 times with sterilized water and the final concentration was adjusted to 10^6/ml.

Staining and microscopy of stomata

Control and infected leaves with *A. brassicae* were detached from seedlings, stained with different dyes (Table 1) and analysed by light and fluorescent microscopy. Opened and closed stomata from 5 areas in 10 leaves from different seedlings were counted. The microscopical views shown in this paper are based on three independent biological experiments. Stomata are considered as closed when no open space can be visualized between the two guard cells.

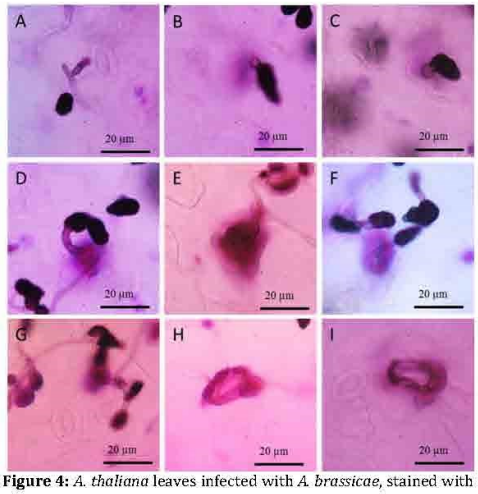

Figure 4: *A. thaliana* leaves infected with *A. brassicae*, stained with Fuchsin acid and observed under a fluorescent microscope at channel 365 nm (A, B, C); mycelium penetration through the aperture (D, E, F); colonization of guard cells (G, H, I).

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Light microscopy

Step 1 Detached Arabidopsis leaves are transferred into 200 µl GL solution on the surface of a glass slide and covered with a glass coverslip.
Step 2 Observation of the samples by light microscopy.

Staining with Calcoflour white

Step 1 Arabidopsis leaves are soaked in 500 µl Calcoflour solution for 5 min.
Step 2 Stained leaves are transferred into 200 µl GL solution on the surface of a glass slide and covered with a glass coverslip.
Step 3 Observation of the samples by fluorescent microscopy at the 365 nm channel.

Staining with Nile red

Step 1 Arabidopsis leaves are soaked in 500 µl Nile red solution for 10 min in darkness.
Step 2 Stained leaves are transferred into 200 µl GL solution on the surface of a glass slide and covered with a glass coverslip.
Step 3 Observation of the samples under fluorescent microscopy at the 450-490 nm channel.

Staining with Methyl green

Step 1 Arabidopsis leaves are treated with 500 µl KOH (10%) for 2 min.
Step 2 KOH treated leaves are washed in distilled water.
Step 3 Leaves are soaked in 500 µl Methyl green solution for 5 min.
Step 4 Stained leaves are transferred into 200 µl GL solution on the surface of a glass slide and covered with a glass coverslip.
Step 5 Observation of the samples under fluorescent microscopy at the 365 nm channel.

Staining with Fuchsin acid

Step 1 Arabidopsis leaves are soaked in 500 µl Fuchsin acid solution for 5 min.
Step 2 Stained leaves are transferred into 200 µl GL solution on the surface of a glass slide and covered with a glass coverslip.
Step 3 Observation of the samples under fluorescent microscopy at the 365 and 450-490 nm channels.

Staining with Aniline blue

Step 1 Arabidopsis leaves are soaked in 500 µl Aniline blue solution for 5 min.
Step 2 Stained leaves are transferred into the 200 µl GL solution on the surface of a glass slide and covered with a glass coverslip.
Step 3 Observation of the samples under fluorescent microscopy at the 365 nm channel.

5. Discussion

In this study, genetic and biological approaches were used to investigate resistance / tolerance mechanisms of plants against biotic and abiotic stresses. For this aim, Arabidopsis responses to *P. indica* were investigated under different stress levels to characterize the efficiency of the symbiosis. Furthermore, different Arabidopsis mutants were used to inspect plant responses and interspecies crosstalk during the symbiotic interaction under biotic and abiotic stresses. Biotic and abiotic stresses are a constant part of the plant´s natural habitat. To avoid their harms, plants perceive and respond to them in different ways. Mycorrhizal fungi and their interaction with plants are model systems for scientists to investigate symbiosis but they have also serious weaknesses, e.g. the host dependency of the fungi makes interaction studies complicated. To investigate how plants and fungi interact with each other under single and multiple stresses, and how they communicate with each other, the following interaction conditions were studied:

(1) the effect of high doses of *P. indica* on the performance of Arabidopsis seedlings, their phytohormone, ROS, anthocyanin, and gene expression patterns, as well as on Arabidopsis mutants impaired in phytohormone signaling;
(2) the effect of *P. indica* on the expression profile and physiology of Arabidopsis seedlings during and after the establishment of a beneficial symbiotic interaction; and
(3) the effect of different stress levels on the interaction of *P. indica* and Arabidopsis.

I propose:

(1) High doses of *P. indica* do not result in over-colonization of roots or massive induction of plant defense. High fungal doses have only a mild effect on plant performance.
(2) Chemical exudates of *P. indica* induce stress responses and stress-related gene expression in Arabidopsis roots, before a physical contact between the two symbionts has been established. Once a physical contact is established, the stress responses are down-regulated.
(3) Stress is an unavoidable part of the Arabidopsis-*P. indica* interaction. A slight elevation of the stress level increases the benefits of the plant in the symbiosis.

5.1. The effects of high doses of *P. indica* on plant performance

Growth of Arabidopsis seedlings on high fungal doses did not result in over-colonization of the roots (Fig. 3). In addition, plant performance and the activation of stress responses were only moderately affected, while the growth of the plants was reduced (Manuscript I, Figs. 1A and 2). This could be caused by a limited access of the roots to nutrients in the medium because the surface of the medium was entirely covered by fungal hyphae, or the fungus releases compounds, which inhibit growth of the host. Also, the chlorophyll content and the efficiency of the photosynthetic electron transport were only moderately reduced on the dense fungal lawn (manuscript I, Table1), as previously documented by Camehl et al. (2010). Furthermore, we failed to detect a significant increase in H_2O_2/ROS accumulation (Manuscript I, Fig. 4) and the induction of the H_2O_2-inducible *OXI1* gene (Manuscript I, Figs. 5 and 6). The slight up-regulation of the pathogenesis related gene *PAL2* and of anthocyanin (Manuscript I, Fig. 9; Table 2) shows that the plants suffer to some extent (Manuscript I ,Table 1). While exposure of *A. brassicae* to Arabidopsis seedlings results in a dramatic increase in the H_2O_2/ROS levels, *OXI1* gene expression and the expression of other stress- and defense-responsive genes were comparable to those in wild-type, *ein3* or *jar1* seedlings on high fungal doses. This can be due to the inability of *P. indica* to activate plant defense via MAMP production or suppression of the plant defense machinery by the symbiotic fungus as suggested by Jacobs et al. (2011). The JA level increases on the dense fungal lawn, which is consistent with the intuition of *PDF1.2* (Manuscript I, Fig. 8) and results of Lahrmann et al. (2015) . It might cause the low level of root colonization to maintain a balance between compatibility and defense (Gutjahr et al. 2009). There are also reports in the literature that even necrotrophic fungi can inhibit JA-induced defense gene activation to some extent through suppression of the JA/ET-mediated resistance during early stages of the infection (Zhu et al. 2013). Colonization of barley and *Arabidopsis* roots by *P. indica* is a biphasic strategy; evasion and suppression of host defenses by fungal effectors occur during penetration of the hyphae into the roots. At a later phase, *P. indica* is mostly found within dying root cells (Lahmann and Zuccaro, 2012). We suggest that the roots induce only a mild defense and stress response to *P. indica.* This might indicate that the plant controls the level of fungal colonization to stabilize the symbiosis. The symbiosis between Arabidopsis and *P. indica* is a balanced relationship which is controlled by the plant defense system and the fungal ability to suppress the plant defense. Microclimate, nutrition availability and competition between root and mycelium for nutri-

ents can affect the colonization rate in the symbiosis, and lower or equalized fungal levels might be more efficient than high doses. This could be a useful hint for field applications of *P. indica* and other colonizing fungi. Furthermore, the level of host defense response can be a good indicator for the effectiveness of a symbiotic interaction.

We demonstrate that the roots activate defense responses against *P. indica* during root colonization, however the responses are mild. How these responses are down-regulated once a stable symbiosis is established, remains elusive. To address this question, we analyzed conditions in which two partners are co-cultivated on the same petri dish but have not yet established a physical contact to each other.

5.2. *P. indica* effects on the plant: chemical communication and physical interaction

Before a physical contact is established between the two symbionts on an agar plate, the interaction should occur via chemical mediators. They induce ROS via root NADPH oxidases and/or apoplastic peroxidases, induce stomata closure and enhance stress-related phytohormone levels. Symbiotic interaction of bacteria or mycorrhizal fungi with roots induces ROS production in the early stage of interaction (Fester and Hause 2005, Tanaka et al. 2006). We also demonstrate an increased H_2O_2 level during early phases of the Arabidopsis / *P. indica* co-cultivation (Manuscript II, Fig. 2), which is consistent with ROS induction observed for other beneficial microbes (Fester and Hause 2005, Tanaka et al. 2006). In addition, ABA induction and consequently stomata closure were observed before a physical contact was established (Manuscript II, Fig. 1 and 4). ABA-mediated stomata closure is also induced by PstDC3000 and *Bacillus subtilis* FB17 colonization of roots (Kumar et al 2012). PAMP perception by plants results in restriction of microbial invasion due to stomata closure (Sawinski et al. 2013). Furthermore, we showed that volatiles are not involved in ROS production and defense responses in Arabidopsis roots (Manuscript II, Table S 2).

After the establishment of a physical contact between *P. indica* and Arabidopsis roots, ROS production, stomata closure and defense gene activation were reduced to levels found in control plants. This is in agreement with low levels of ROS production, oxidative damage to lipids and membrane electrolyte leakage in AM plants (Estrada et al. 2013, Evelin and Kapoor 2014).

Up-regulation of the *NRT2.5* mRNA level in the leaves 6 days after co-cultivation, i.e. when a physical contact has been established, reflects a slow rate of root to shoot communication, which has been previously shown for the *Phyllobacterium brassicacearum* STM196 strain interacting with plants (Mantelin et al. 2006). *NRT2.5* is a sensitive leaf marker for root colonization by *P. indica*, but its role remains unclear in the Arabidopsis-fungi interaction. Activation of *NRT2.5* in the roots might be a side effect of the plant´s defense response to the fungus at the 6th day after co-cultivation. This is consistent with a balanced plant defense during symbiosis (Gutjahr et al. 2009). Another possible explanation for the mild defense response at the 6th day of interaction might be that the colonized plant cells start to die (Lahmann and Zuccaro 2012) which might be a direct effect of the fungus.

We showed that ABA plays an important role in the Arabidopsis / *P. indica* interaction, which is in agreement with previous studies in barley (Ballaré 2011) and tomato (Martín-Rodríguez et al. 2011). Elevated ABA levels during early phases of the interaction are consistent with the high number of closed stomata in leaves (Figure 4, manuscript II). High levels of ABA and stomata closure occur in response to biotic stress (Sawinski et al.2013). ABA is essential during full AM colonization and arbuscules development, and its lack results in lower level of AM colonization in tomato (Herrera-Medina et al. 2007).

JA plays an important role in plant-microbe symbiotic interactions (Sun et al. 2006, Rosas et al. 1998, Mabood et al. 2006). JA, JA-Ile and OPDA are involved in plant defense responses (Ballaré et al. 2011) but there is no clear evidence about their role in beneficial plant-microbe symbioses (Gutjahr et al. 2009). Also enhancement of JA level and its catabolism in *P. indica* colonized roots has been reported. (Lahrmann et al. 2015). Elevated JA levels during early phases of co-cultivation are consistent with microarray data that show up-regulation of JA-regulated genes.

Reduction of JA, JA-Ile and OPDA levels six days after co-cultivation, when the stressful phase for the plant is terminated and co-cultivation of the two partners has been established, is comparable with the root / *Mesorhizobium loti* interaction, where these hormones are also down regulated after nodule formation (Kouchi et al. 2004).

Increase of the SA level in roots and shoots during early phases of the Arabidopsis-*P. indica* interaction (Fig. 4, manuscript II) resembles early stage of AM colonization (Blilou et al. 1999). SA is also involved in the plant response to biotrophic pathogens (Lu, 2009). Furthermore

P.indica interaction with Arabidopsis results in accumulation of SA in plant roots (Lahrmann et al. 2015).

There are clear differences between early and later time points with regard to the regulation of defense related genes. Moreover, some defense related genes are up-regulated during both time points but their regulations are clearly lower six days after co-cultivation compared to two days. Those stress- and defense-related genes which are still highly expressed six days after co-cultivation, might be important for restricting colonization (Sherameti et al. 2008, Camehl et al. 2010). Up-regulated genes during the early and late phases of the Arabidopsis-*P. indica* interaction may play a role in compatibility of the partners as it has been proposed by Güimil et al. (2005). Suppression of the plant defense system might be required for colonization of Arabidopsis roots by *P. indica*, in agreement with findings with AMF (Campos-Soriano et al. 2010) and *P. indica* symbiosis with barley (Jacobs et al. 2011).

It is possible that the defense related genes, which are regulated at later time points, are important regulators of plant / fungus interaction or involved in compatibility. Those genes that are repressed by *P. indica* during later phases of the interaction, might have a different function and might also be differently regulated by suppression mechanisms. The balanced and differentiated activation of certain defense genes during different phases of the development of the symbiosis might be a result of co-evolution between the two partners, as proposed by Hoeksema (2010).

The initial stimulation of ROS production by fungal exudate compounds might trigger ROS-activated defense genes and processes, which might differ from those activated during later phases of the symbiosis. One might speculate that these two types of stress responses might fulfill different functions, and this might be related to the remodeling of the root and mycelium architectures during the establishment of the interaction.

5.3. The role of stresses in Arabidopsis/*P. indica* symbiosis

How a plant reacts to biotic and abiotic stresses and how they recognize beneficial and non-beneficial microbes are important questions in understanding its response to beneficial microbes, and environmental changes. This helps scientists to design ecofriendly plans to increase plant´s yield and to fulfill human food requirements in future.

Discussion

We found better performance of colonized Arabidopsis plants under different mild stress conditions: nutrient limitation, heavy metal, light and osmotic stress, salinity, and exposure to pathogen attack. Our results confirm several studies of root-colonizing microbes on crop productivity under stress (Nadeem et al. 2014, and references therein). Mycorrhizal plants are more resistant to heavy metal stress than non-mycorrhizal plants (Schützendübel and Polle 2002, Repetto et al. 2003), e.g. by accumulation of toxic metals in fugal mycelium or fungal metal chelators (Khan et al. 2000; Ahonen-Jonnarth et al. 2000), activation of fungal antioxidant processes (Abdel 2011), or biosynthesis of nano- and micro-particles for scavenging and reduction of metal ions in the root environment (Vahabi et al. 2011 and Feng et al. 2013). Osmotic stress tolerance induced by AMF against NaCl has been described by Augé et al. (2014) which is consistent with our findings. It has been shown that ROS levels increase under high light, while *P. indica* counteracts light stress by activating the antioxidant system (Baltruschat et al. 2008; Vadassery et al. 2009). *P. indica* enhances plant performance systemically. With increase in the light intensity, also the ROS level and subsequent damage to the leaves increase. *P. indica* colonized plants suffer less than control plants under high light intensity (Vadassery et al. 2009). This can be caused by a systemic enhancement of the antioxidant capacity of the plant, as suggested by Vadassery et al. (2009). Furthermore, like in our studies, *P. indica* and other beneficial microbes promoted plant growth after exposure to metals with different toxicity levels (Schützendübel and Polle 2002, Repetto et al. 2003, Vahabi et al. 2011; Feng et al. 2013). This might occur directly via detoxification in the root environment by fungal mycelia or by secondary effects of the fungi through an increase in the antioxidant potential of the plant, as previously discussed.

The physical structure of the fungi and their connection to the roots are critical for nutrient uptake. *P. indica* colonization results in altered root architecture and their parameters improve according to criteria described in Galkovskyi et al. (2012). P limitation stimulates AM symbiosis and Pi and N acquisition (Bonneau et al. 2013, and references therein). Limitation of PO_4^{3-} (Bolan et al. 1987), NO_3^- (Jin et al. 2012) and SO_4^{2-} (Gahan and Schmalenberger 2014) enforces AM interaction with their hosts. Since root colonization increases under our experimental conditions with increase in the shortage of the available nutrients, this might play a crucial role in the promotion of plant performance. This also results in the down-regulation of *PR* genes. Smith and Smith (2012) showed that PO_4^{3-} and NO_3^- rich soil reduces AM root colonization. Similarly we demonstrated that rich media (PNM 1x and 4x) restricts the level of fungal colonization and con-

sequently reduces the *ITS* mRNA level in roots. These inhibitory effects are also reflected by the reduced growth rate of plants in nutrient-rich media (Fig. 3, manuscript III). The plant defense system might produce anti-fungal compounds to restrict root colonization through up-regulation of the *PR* genes under high nutrition condition. Accordingly, it is fair to suggest that the level of biotic interaction between the two symbionts is controlled by the nutrition status.

Pi uptake in AM symbiosis is a mixture of direct root / root hair and fungal uptake (Smith et al. 2011). Depletion zones resulting from nutrient uptake represent stress and control over this problem was gained by evolving different mechanisms by the plants, e.g. association with mycorrhizal fungi (Marschner, 1995). Whether Pi uptake is responsible for *P. indica*-mediated growth promotion has been discussed intensively (Barazani et al. 2005, Achatz et al. 2010, Shahollari et al. 2005, Yadav et al. 2010). Higher concentration of Pi in the media reduces Pi-uptake relative to the control (Bakshi et al. unpublished, Yadav et al. 2010, Kumar et al. 2011). It can explain our findings in higher concentration of the media. There might be a synergistic effect between low Pi concentration (Bakshi et al. unpublished, Yadav et al. 2010, Kumar et al. 2011) and low root colonization in high nutrient concentrations which results in reduction of plant growth. *P. indica* and plant Pi transporters can play a role in this process. The high-affinity Pi transporter, PiPT, of *P. indica* participates in growth promotion by transferring Pi to the host (Yadav et al. 2010, Kumar et al. 2011). Therefore, reduction of fungal mycelia in higher nutrient concentrations may reduce the fungal potential of transferring Pi to the plant. In Arabidopsis, WRKY6 has been shown to be a central regulator of Pi uptake and metabolism under Pi-limiting conditions. WRKY6, -42 and -75 are regulators of nutrient homeostasis like Pi (Rushton et al. 2010, Chen et al. 2009), and WRKY6 is involved in regulation of plant senescence, pathogen induced *PR1* expression, boron starvation and low Pi stress responses (Robatzek and Somssich 2002, Chen et al. 2009, Kasajima et al. 2010). Furthermore, repression of *PHO1*, for a plasma membrane protein involved in Pi loading into the xylem, is an important function of WRKY6 (Chen et al. 2009). Better performance of the *wrky6* mutant under Pi limitation and *P. indica* colonization (M. Bakshi, personal communication) is consistent with Chen et al. (2009). Several mechanisms have been proposed to modulate root system architecture in response to low Pi conditions, including changes in auxin transport, increase in sensitivity to auxin (López-Bucio et al. 2002, 2005, Nacry et al. 2005, Pérez-Torres et al. 2008) and expansin-dependent cell division (Guo et al. 2011). Microarray analysis by M. Bakshi in our laboratory (unpublished) indicates that inactivation of

WRKY6 results in induction of auxin-regulated genes (include SAUR genes) under Pi deprivation in *P. indica*-colonized seedlings. SAUR proteins are involved in cell expansion via auxin transport stimulation (Kong et al. 2013) and thereby contribute to the root development. Bakshi et al. (unpublished) proposed a synergistic effect of WRKY6 and *P. indica* on plant performance under Pi limitations. PHO1 is a central regulator in this scenario and participates in Pi transport from root to shoot by loading Pi into the xylem vessels (Hamburger et al. 2002, Chen et al. 2009). In summary, we demonstrate that *P. indica* promotes nutrient uptake and enhances tolerance under stress conditions. This suggests that *P. indica* effects cannot be replaced by genetic modifications in plants.

The root architecture is influenced by *P. indica* (Peškan-Berghöfer et al. 2004, Dong et al. 2013, Ye et al. 2014) and this has been correlated with growth promotion in *Brassica campestris* cv. Chinensis (Chinese cabbage) (Dong et al. 2013). Furthermore, an increased auxin level in the roots was found in colonized Chinese cabbage roots (Lee et al. 2011). Nutrient limitations induce lateral root formation after colonization by AMF (Harrison et al. 2002, Javot et al. 2007, Kobae and Hata, 2010, Yang et al. 2012, Gutjahr and Paszkowski 2013). Thus, alteration of the root architecture by *P. indica* colonization can be caused by nutrient depletion in the root environment or induced by elevated auxin levels.

Overall, according to our and other results, *P. indica* interaction with Arabidopsis results in more benefits for the plant under higher level of stress. Furthermore, the root colonization level by the fungus correlates with the stress level. Fungus-induced benefits for the plants are based on more than one effect including physical structure of fungi, root architecture, transcriptomic changes, plant hormone alterations, as well as physiological and synergistic effects between the symbionts.

5.4. Defense dynamic of Arabidopsis/*P. indica* symbiosis

Defense genes in the interaction of *P. indica* with Arabidopsis roots are activated under different conditions. To understand the beneficial and non-beneficial effects of the fungus on Arabidopsis, we used high fungal doses in order to stimulate biotic stress and to find limitation borders in the symbiosis of *P. indica* with plants. Also, we investigate plant gene expression pattern induced by fungi during short and long term interaction to see changes.

Discussion

Many plant defense-related genes are up-regulated during Arabidopsis / *P. indica* interaction (Campos-Soriano and Segundo 2011, Campos-Soriano et al. 2010, Jacobs et al. 2011). Activation of the plant defense system can be the consequence of an increased demand for sugars by the fungus, which plays a role in the stabilization of root colonization by AM fungi (Campos-Soriano and Segundo 2011). Besides the growth and biomass enhancement, *G. intraradices* induces plant defense genes in rice, as well as PR proteins and antioxidant enzymes. These results show the capacity of AM fungi to circumvent the plant immune system (Campos-Soriano et al. 2010), as also described for the *P. indica* / *Arabidopsis* interaction by a broad-spectrum suppression of innate immunity in roots (Jacobs et al. 2011).

We demonstrate that *P. indica* derived exudates activate stress and defense responses in Arabidopsis roots and shoots prior to a physical contact between the partners (at early time point, 2 days after co-cultivation). Once a physical contact is established (later time point, 6 days after co-cultivation), defense-related genes are down-regulated. Lower expression of defense related genes at the later time point indicates that the fungus can deal with the host´s defense machinery, which might result in lower root colonization levels, in particular at higher nutrient concentrations. This could play a role in stabilizing the symbiosis and the colonization level, e.g. by restricting root colonization (Sherameti et al. 2008, Camehl et al. 2010). The important role of the plant in controlling the colonization level of the fungus is demonstrated by the different colonization rates in different media and the restricted fungal growth on the dense fungal lawn.

Moreover, comparison of the available microarray data in our laboratory for colonized Arabidopsis roots at different time points after co-cultivation (2 and 6 days after co-cultivation) in my studies and 3 days after infection in a study performed by Bakshi et al. (unpublished) shows that there are commonly regulated genes. They are mainly related to biotic and abiotic stresses, signaling, cell wall biosynthesis, oxidation and peroxidation. These genes probably play a role in stabilizing the beneficial interaction and control of root colonization (Sherameti et al. 2008, Camehl et al. 2010). Moreover, the “6 days” dataset is quite different from the other two and expression level of defense genes decreases after long term interaction.

5.5. Biotic stresses effect on Arabidopsis/*P. indica* symbiosis

P. indica has been known as a protecting fungus against leaf, root and stem pathogens such as *Blumeria graminis* f. sp. *tritici*, *Fusarium culmorum*, *Fusarium verticillioides*, *Pseudocercosporella herpotrichoides, Golovinomyces orontii* via local or systemic disease resistance (Molitor et al. 2011, Stein et al. 2008, Kumar et al. 2009, Serfling et al. 2007, Fakhro et al. 2010). Alternaria infection of *Arabidopsis* leaves resulted in ROS production. In *P. indica*-colonized plants, ROS level reduced. This might be caused by ROS scavenging effects, since *P. indica* stimulates antioxidants in the leaves (Baltruschat et al. 2008, Vadassery et al. 2009). In addition, the root-associated fungus promotes the performance of the aerial parts systemically via signals from the roots. We introduced the new and highly sensitive biophoton technology to measure the amount of ultra-weak photon emission inside plant leaves. This technology detects ultra-weak emission of photons originated mainly from the ROS generation and metabolic activities as by-products of cellular respiration (Takeda et al. 2004, Kobayashi 2014). Biophoton measurements have been used for demonstration of stress conditions and it is a good indicator of ROS level inside the cell (Takeda et al. 2004, Wijk et al. 2013, Kobayashi 2014). We used this technology as appropriate technique to describe plants stress quantitatively, and our results indicate that it is a reliable tool to measure plant fitness under different physiological conditions. *P. indica* interaction with roots reduces the biophoton level in infected and non-infected seedlings with *Alternaria* spores. This confirms the lower ROS level in colonized plants. Additionally, beneficial effects of *P. indica* have also been shown by growth promotion and regulation of stomata aperture. These results are consistent with the biophoton measurements and indicate the systemic protective role of *P. indica* against Alternaria leaf infection.

Alternaria brassicae infection results in the accumulation of defense phytorhomones in shoots, and this is repressed by *P. indica*. In summary, we found that *P. indica* diminishes Alternaria-induced biotic stress. These results demonstrate that stress within a reasonable and non-toxic scale stimulates the symbiotic interaction, which in turn results in better performance of the plant.

An inhibitory effect of *P. indica* on the vascular root colonizing pathogen *V. dahliae* in Arabidopsis seedlings has been shown by Sun et al. (2015) (in manuscript IV). The authors showed that Arabidopsis seedlings pretreated with *P. indica* performed better after *V. dahliae* infection

than the controls. The pretreated seedlings showed comparable biomass and total chlorophyll as the mock-treated control. In addition, treatment with *P. indica* prior to the infection by the pathogen reduced stress-related phytohormones accumulation and *PR* gene expression, increased stomata opening and inhibited the spread of *V. dahliae* hyphae in the shoots and roots (Sun et al. 2015, manuscript IV). *EIN3* plays an important role in development of *Verticillium* pathogenicity and the *ein3* mutant is more resistant against disease. Pantelides et al. (2010) showed that ET via the receptor ETR1 is required for *V. dahliae* infection in *Arabidopsis* that is consistent to Sun et al (2015 manuscript IV). Furthermore, they reported that *V. dahliae* infection in *ein3* mutant produces higher ET level compared to the wild type. This can be caused by an induced defense response mediated by the *EIN3* mutation (Sun et al 2015, manuscript IV).

Figure 4 summarizes my results in a simple scheme. I have investigated effect of different stresses (abiotic and biotic) on *A. thaliana-P. indica* interaction. Stress plays an important role in forming symbiotic interaction between these partners and a mild enhancement of stresses results in strengthening of fungi beneficial effects on plants.

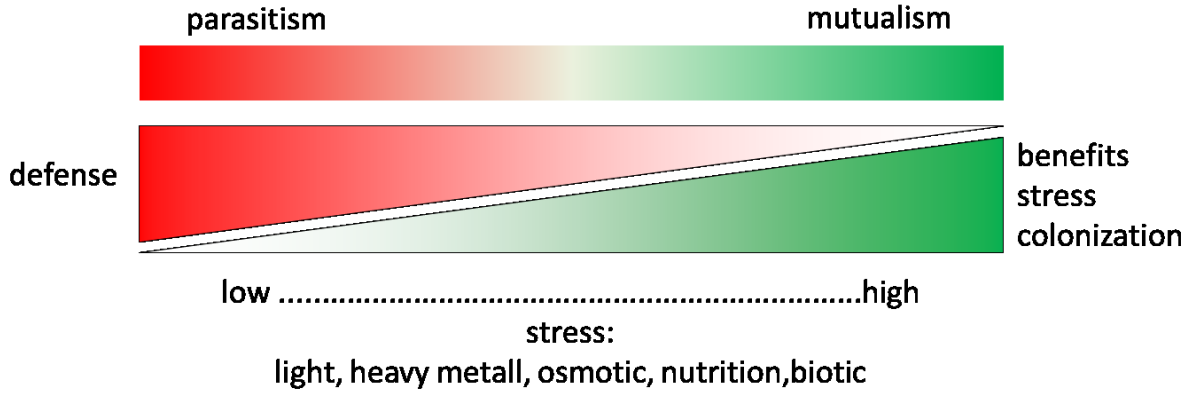

Fig 5.1. Model of stress role in Arabidopsis/*P. indica* interaction.

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7. Appendix

7.1. Declaration

I declare in accordance with the conferral of the degree of doctor from the faculty of biology and pharmacy of the Friedrich Schiller University, Jena that the submitted thesis is written only with the assistance and literature cited in the text.

This thesis has not been previously submitted either to the Friedrich Schiller University Jena or to any other university.

Jena (May 2015) ----------

(Khabat Vahabi)

7.2. Curriculum vitae

Personal Details

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University education:

(2002) Bachelor degree: Plant Protection Bu-Ali Sina University, Hamedan, Iran.

Areas of Concentration: Pathogenic Fungi

Minor: Plant pathology

(2005) Master of science: Agricultural Biotechnology, Isfahan University of Technology, Isfahan, Iran.

Thesis: "Diversity of *Trichoderma* spp. Related to Button Mushroom (*Agaricus bisporus*) Using Microscopic, Molecular and Morphological Approaches.

Areas of Concentration: Microscopy and Genetic Diversity

Minor: Molecular Markers

7.3. List of publications

Vahabi K, Sherameti I, Bakshi M, Mrozinska A, Ludwig A, Oelmüller R, (2015) Microarray analyses during early and later stages of the Arabidopsis/Piriformospora indica interaction Genomics Data, 6:16–18

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Sun C, Shao Y, **Vahabi K**, Lu J, Bhattacharya S, Dong S, Yeh K-W, Sherameti I, Lou B, Baldwin I T, Oelmüller R (2014) The beneficial fungus *Piriformospora indica* protects Arabidopsis from *Verticillium dahliae* infection by downregulation plant defense responses. *BMC Plant Biology* 14:268.

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